AN ELECTROPHYSIOLOGICAL EXPLORATION INTO THE SERTOLI CELL, UTILIZING PLANT EXTRACTS WITH A VIEW TO CONTRACEPTION

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ABSTRACT

AN ELECTROPHYSIOLOGICAL EXPLORATION INTO THE SERTOLI CELL, UTILIZING PLANT EXTRACTS WITH A VIEW TO CONTRACEPTION

Certain medicinal plant extracts have been shown to have a contraceptive effect on male rats (Rajasekaran *et al.*, 1988) and mice (Kong *et al.*, 1986). A possible site for the mechanism of action has been identified as the testis. The Sertoli cells within the seminiferous tubules of the testes are of fundamental importance to the developing sperm. These cells create a unique environment, which, without, would lead to the cessation of spermatogenesis (Gow *et al.*, 1999). Knowledge of the Sertoli cell is thus essential in understanding the control of developing germ cells within the seminiferous tubules of the testis.

The first part of this study made use of the electrophysiological method of intracellular punctures to determine the induced potential (*Vi*) caused by administered channel blockers and the effects of the extracts used in the antifertility study on the membrane transport mechanisms.

The TM4 Sertoli cell line was cultured in bicameral chambers as endorsed in previous literature. This method of cell culture allowed the *in vivo* environment of the seminiferous tubule to be mimicked. More specifically, this culturing technique allowed for the apical membrane to be measured independently from the basolateral membrane, and allowing for the TM4 Sertoli cell to be mapped with reference to membrane ionic pumps and channels. It further served as an *in vitro* model for measuring the developing tight junctions of the blood-testis barrier.

Histology of TM4 Sertoli cells grown on matrigel filters in the presence of hormone-supplemented media showed that the cells grew into confluent monolayers within 24 hours. These cells were cuboidal like in shape and the monolayer only lasted for 48

hours, whereafter it became a multilayer. In comparison, cells grown in unsupplemented media did not develop into confluent monolayers and were found to resemble squamous epithelia. When compared with previous literature, it was found that the TM4 Sertoli cell differs to primary cultures with respect to growth rate and cell morphology.

This study also endorsed the view that hormone supplementation in media is important for the formation of tight junctions between adjacent Sertoli cells. This was established by measuring the transepithelial potentials of cell cultures exposed to supplemented media and cell cultures grown in unsupplemented media.

Intracellular studies revealed that the basolateral membrane differs significantly in electrical potential from the apical membrane ($P<0.001$). The basolateral membrane consistently measured at –47.46±1.74 mV whereas the apical membrane measured at –21.67±1.92 mV.

Bumetanide and furosemide, inhibitors of the $Na^+K^+2Cl^-$ symport, brought about a depolarization in intracellular potential when added to either compartment of the bicameral system. This gave evidence to the existence of this symport in both membranes.

Cells exposed to a chloride free environment also brought about a depolarization in intracellular potential, indicating that the above mechanisms of chloride transport are important in the preservation of intracellular potential.

Treatment of the cells to $BaCl_2$, a blocker of potassium channels, had no effect on the intracellular potential when administered to either compartment. These results indicate that this blocker has no effect on cultured TM4 Sertoli cells. However, this does not disprove the existence of these channels in Sertoli cell membranes, as cells exposed to potassium free Ringers depolarized. These results merely indicate that barium sensitive channels are not found in the TM4 Sertoli cell.

Depolarization of the intracellular potential resulted from the introduction of acetazolamide, an inhibitor of carbonic anhydrase. This effect was noted with apical as well as basal administration, indicating the presence of this enzyme within the Sertoli cell as an intracellular potential regulator.

Amiloride, a blocker of sodium channels hyperpolarized the cells upon introduction to the apical and basal media respectively, indicating the presence of sodium channels in both membranes. Exposure of the cells to sodium free Ringers led to a hyperpolarization of the cells, backing up the data generated from amiloride exposure.

These preliminary studies have indicated the presence of specific membrane channels and pumps in the TM4 Sertoli cell. They provide a useful base for future investigation involving contraception in male mice as well as the ionic transport of TM4 Sertoli cells. Furthermore, these studies have provided novel electrophysiological data for the TM4 Sertoli cell line and have established sound techniques for studying Sertoli cells *in vitro*.

The second part of this study investigates the *in vivo* effects of crude extracts derived from plants that have been shown to produce a male contraceptive (Mdhluli and Van der Horst, 2002), and uses *in vitro* tools to identify a mechanism of action as noted in the *in vivo* study.

The plant species of *Olea* indigenous to the Western Cape region of South Africa was collected and made up into a crude extract. The extract is known to possess oleanolic acid, a contraceptive compound known to be present in the olive plant.

An antifertility and toxicology investigation performed in this study endorsed the view that the crude extracts derived from the *Olea* species have a contraceptive effect in male albino mice without subjecting the mice to toxic side effects. This study showed that oleanolic acid caused a complete inhibition of fertility in male mice, a 66% inhibition of fertility in male mice subjected to a crude extract made from *Olea europaea* and a 33% inhibition of fertility in male mice subjected to a crude extact derived from *Olea*

exasperata. Histology of the liver and the testes revealed that there were no toxic effects caused by the administration of the extracts to the animals, but haematological analysis tends to indicate that with longer periods of extract administration, the immune system may be compromised in animals treated with *Olea europaea* extract.

A 24 hour administration of an oleanolic acid extraction 0.1% ethanol from the leaves of *Olea europaea* and *Olea exasperata* was carried out to investigate the effect of these extracts on the developing blood-testis barrier *in vitro*. Cultured TM4 Sertoli cells grown on a matrigel surface in a bicameral system to a confluent monolayer have been shown to mimic the in vivo blood-testis barrier (Onoda *et al.*, 1990). Measurement of the transepithelial resistances of the TM4 Sertoli cells were shown to indicate the status of the tight junction formation between adjacent TM4 cells (Janecki *et al.*, 1990). This revealed that the developing junctional complexes between adjacent Sertoli cells were broken down by the administration of these extracts. This clearly showed that these extracts compromise the blood-testis barrier.

The above extracts, however, had no effect on the intracellular potential in the TM4 Sertoli cell. This indicates that the mechanism by which these extracts compromise the blood-testis barrier is not by incapacitation of cells, but by interfering with the formation of junctional complexes between adjacent Sertoli cells.

Conclusion

This study provided novel data to the electrophysiological status of the TM4 Sertoli cell. Furthermore, OA data presented new data as to the mechanism whereby it brings about its contraceptive action and also provides an explanation as to why the testes can return to normal spermatogenesis following the removal of OA. This study endorses the view that OA is an excellent candidate as a reversible male contraceptive.

DECLARATION

I, Faisel Mosoval, student number 9633938, hereby declare that ***An Electrophysiological Exploration Into The Sertoli Cell, Utilising Plant Extracts With a View to Contraception*** is my own work, that it has not been submitted for any degree or examination at any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references

Full name: Faisel Mosoval ***Date:*** March 2003

Signed:

CONTENTS

GENERAL INTRODUCTION

The use of plants in medicine has been an ancient practice dating back to early man. Plants were the primary source of medicinal remedies, the knowledge passed down from generation to generation. Then Europe entered the Dark ages. During this period of time, most of this natural medicine was forgotten by all except for monks who cultivated medicinal plants which in turn, sustained the suffering of mankind (Phillipson, 2000). The 19th century saw the development of modern medicine and pharmacy. Scientists started isolating certain active components of medicinal plants. This led to the discovery of many drugs commonly used today.

South Africa has overwhelmingly rich plant diversity, boasting over 30000 species of flowering plants (Van Wyk *et al.*, 1997). This accounts for at least 10% of the world's higher plants. The Cape floral kingdom has approximately 9000 species, making this region the most diverse template on earth. At least 3000 species are used for their medicinal value. Examples of this medicinal values are plant derived medicines including aspirin, atropine, codeine, morphine and even the anti cancer drug, taxol.

The South African market for medicinal plants is a booming industry. Medicines are derived from plants on a day-to-day basis, resulting in large volumes of plants being cultivated, harvested and sold. This has a positive contribution towards the economy of the country, stimulating informal and commercial sectors, bringing with it job creation, poverty relief and a safe and healing form of natural medicine.

There is, however, a lack of detailed documentation of the uses of medicinal plants and the methods in which they are prepared. More exclusively, there is a lack of scientific evidence to substantiate the claims made by previous generations on the efficacy of medicinal plants.

To date, Chinese scientists are the leading researchers into the field of medicinal plants. With a wide area of focus on ethnomedicine and their pursuit in finding fertility

regulating agents from this ancient natural practice. Many discoveries, ranging from anti-implantation agents to abortifacient and pregnancy termination have been the outcomes of such studies. These studies have also led to the discovery of a male contraceptive (Kong *et al.*, 1986) abundant in many plant species, which was found to exhibit antifertility in mice and in rats (Rajasekaran *et al.*, 1988). In this study an investigation is made into the toxicological effects as well as the possible antifertility effects of crude extracts from the Oleaceae, a family of plants known to possess this contraceptive compound (Garcia-Garnados *et al.*, 1998).

A site for the possible action of this contraceptive has been identified as the seminiferous tubules of the testis. It is the cells of these tubules that form the main focus of this study.

The Sertoli cells housed in the seminiferous tubules of the testis are the most important factors for regulating the development of spermatozoa. It is generally accepted that these cells form tight junctions with each other, forming the ever-important blood-testis barrier. It has been postulated that the Sertoli cells create a unique and essential microenvironment for the developing spermatozoa. These cells serve as a target for secreted hormones, produces proteins essential for germ cell development and forms the blood-testis barrier, preventing the destructive antibodies in the blood from destroying the developing spermatozoa (Berne and Levy, 2000).

The *in vitro* component of this study makes use of the electrophysiological techniques of intracellular recordings and transepithelial resistance measurement of the cultured Sertoli cell. These studies attempt to reflect the mechanisms of ion transport and the possible mechanism of action of crude extracts and a potential male contraceptive, oleanolic acid on Sertoli cells in culture. This study has given novel information on the TM4 Sertoli cell and is the first to independently measure the electrophysiological characteristics of membranes of the Sertoli cell independently. The identification of certain pumps and channels in each membrane of the TM4 Sertoli cell has also been carried out.

Using this methodology, the effects of pure oleanolic acid and crude extracts containing oleanolic acid were monitored to establish a mechanism of action and to identify whether action of the crude extracts of selected plants was masked in any way.

Chapter 1 gives a general background into the Sertoli cell and attempts to give an insight as to the work that has been done on the cell this far.

Chapter 2 reports on an antifertility and toxicological screening of the extracts used in the study. It serves as motivation behind using these extracts in the electrophysiological studies. This chapter has been written up in the form of an article for *The Journal of Andrology*.

Chapter 3 gives a detailed description into the cell culturing, apparatus, solutions drugs and electrophysiological techniques used in this study. The importance of the above protocols is discussed here.

Chapter 4 reports on the electrophysiological experiments performed on the cell cultures. This is followed by chapter 5, which discusses the outcomes of the experiments and concludes the findings of this study.

All the references in this study follows chapter 5, along with the appendices.

CHAPTER 1

LITERATURE REVIEW: THE SERTOLI CELL

1.1 Introduction

This review summarises recent developments in the understanding of the role of Sertoli cells in testicular function. Although a range of mammalian species has been studied, the majority of research has been exercised on rodents, which will be focused on in this review and research project.

The seminiferous tubules of the testis comprise mainly of two cell types, the Sertoli cells and germ cells (Tortora and Anagnostakos, 1987). Sertoli cells make up approximately 38% of the total cell population of the seminiferous tubule (Verhoeven, 1999). This gives an indication of the huge spermatogenetic potential of the Sertoli cell, as each is able to nurse 4 elongated spermatids in primates and 12 in the rat (Verhoeven, 1999).

The Sertoli cells fulfil many functions, the most prominent being the provision of co-ordinated support, maintenance and nutrition for developing germ cells (Thibodeau and Patton, 1996). Sertoli cells closely co-ordinate spermatogenesis by releasing essential proteins, peptides and steroids (Jegou, 1992). To add to this, the Sertoli cell is also responsible for protecting the seminiferous tubule from protease damage. This is achieved by the synthesis and secretion of protease inhibitors by the Sertoli cell (Cheng *et al.*, 1990).

Sertoli cells develop tight junctions to form a selectively excluding barrier, called the Blood-testis Barrier (BTB). This is an essential function of Sertoli cells, which without, spermatogenesis is halted. An additional function is the regulation of the luminal environment. This luminal environment is in conjunction in particular to the testis and is essential for spermatogenesis (Setchell, 1979). For this reason, it is essential to review the ion transport across the Sertoli cells.

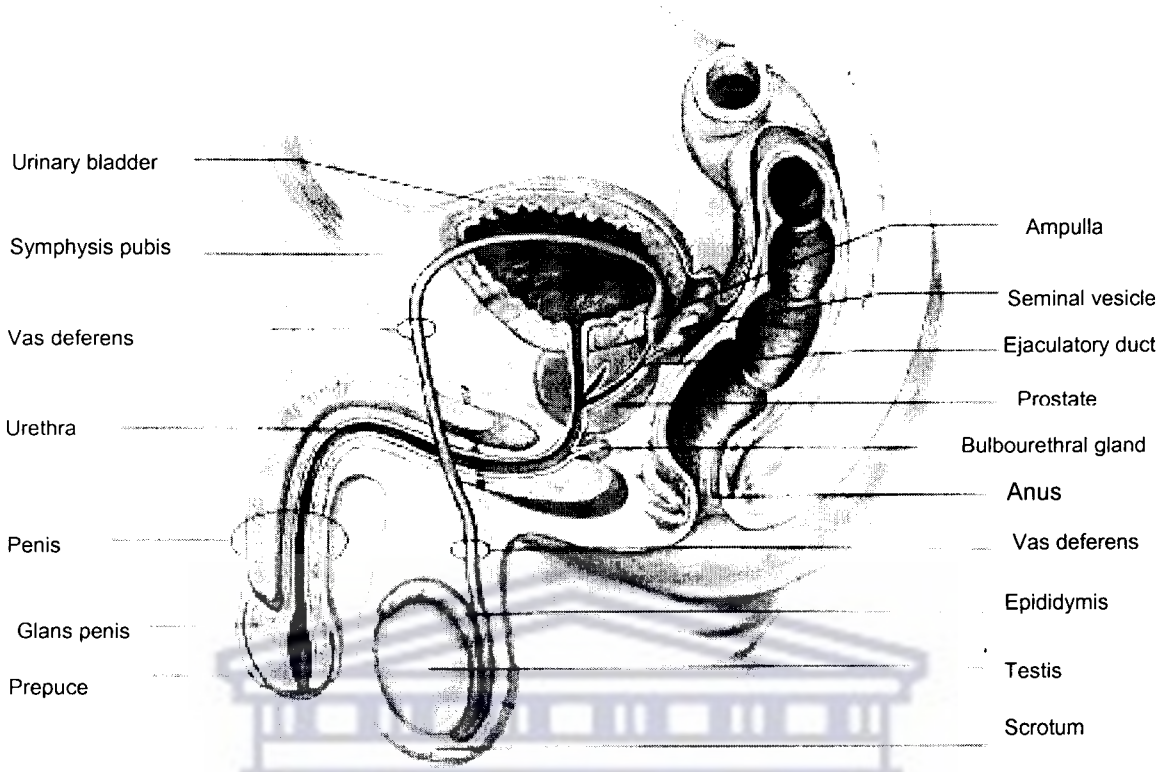

Figure 1.1 Diagram of male anatomy indicating the location of the testes. Taken from Thibodeau and Patton, 1996.

1.2 The role of Sertoli cells in the blood-testis barrier

1.2.1 Morphology of the Sertoli cell

The Sertoli cell is a somatic element within the seminiferous tubule that extends from the innermost layer of the basement membrane lining the seminiferous tubules into the lumen. These Sertoli cells have been noted to contain very little heterochromatin when compared to other somatic cells. This is due to the large amounts of euchromatin in the nucleolus, which is expressed in accordance with the highly versatile functions of this cell (Bardin *et al.*, 1994).

The Sertoli cells are linked by specialised junctions, which are thought to provide intercytoplasmic pathways for diffusing molecules, and are found at all developmental stages (Jegou, 1992).

The Sertoli cell secretes a potassium-rich fluid (40-50mM) into the lumen of the seminiferous tubule (Waites and Gladwell, 1982). This fluid is unique for it bathes the spermatozoa in a microenvironment essential for survival (Fisher, 2002).

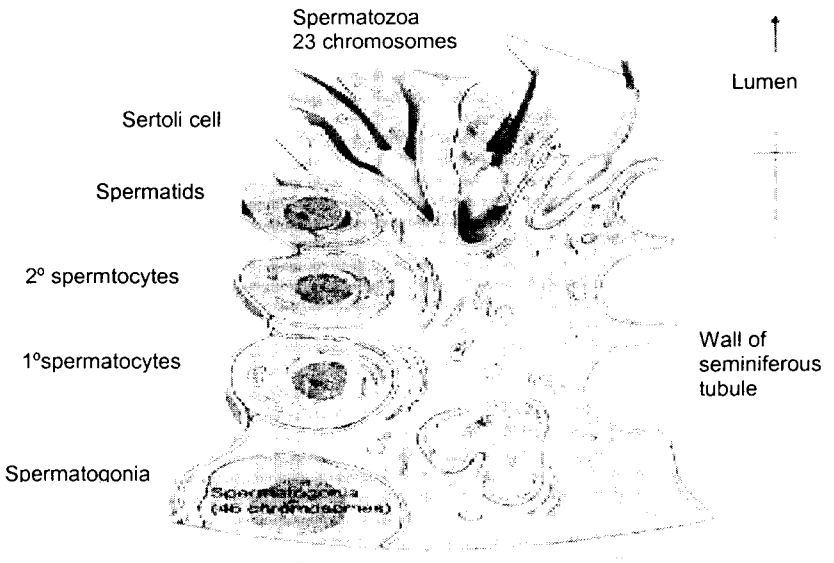

Figure 1.2. Diagram showing the different stages of germ cell development.

Numerous primary lysosomes, autophagic and heterophagic vacuoles are believed to participate in the phagocytosis and digestion of germ cells that degenerate during spermatogenesis (Bardin *et al.*, 1994). In this way, half of the potential elongated spermatids are degraded to yield energy in the form of ATP to drive the metabolic processes of the other half. It is assumed that the spermatids located more closely to the lumen are pinched off by the cytoplasm of the Sertoli cell to become spermatozoa. The energy is provided by the degradation of the spermatids on the apical membrane.

The structure of the multiple Golgi elements found in the Sertoli cell indicates a highly active mechanism for processing newly synthesized proteins. However, there seems to be insufficient rough endoplasmic reticulum in the cells, a lack of large vacuoles and very few vesicles opening to the lateral surfaces of the Sertoli cell. This could indicate that the Sertoli cells are capable of producing proteins that are secreted immediately after synthesis.

The immature Sertoli cells are irregular in shape and their cytoplasm contains few mitochondria, a small Golgi complex and elements of endoplasmic reticulum. Following the establishment of inter-Sertoli cell tight junctions. At puberty, the cells become larger and undergo differentiation in the cytoplasmic processes between germ cells. Organelles develop and numerous modifications in patterns of nuclear arrangement occur in the maturing cell.

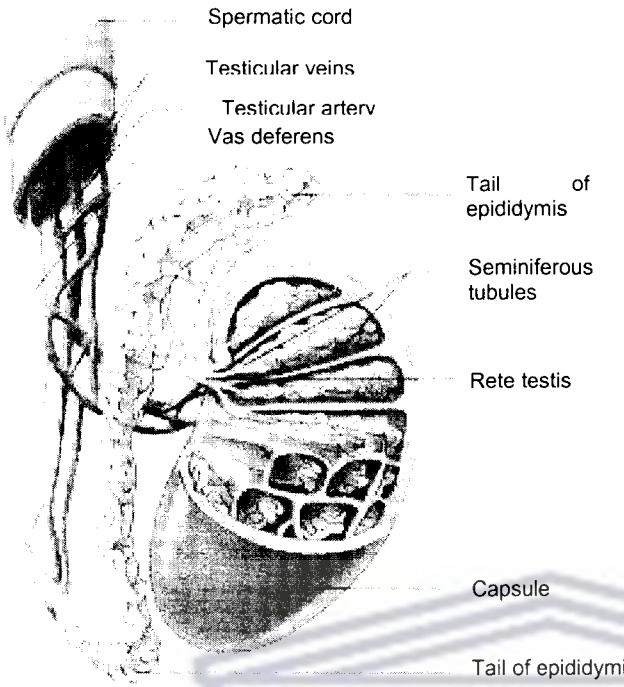

Figure 1.3. Diagram representing the seminiferous tubules. Taken from Fox (1996).

Thirty-five days after birth, rat Sertoli cells are regarded as mature. Adult rat Sertoli cells are noted to possess large and irregular nuclei, situated in the basal portion of the cell. These nuclei are characterised by a homogenous nucleoplasm, which primarily contains euchromatin and a distinctive tripartite nucleolus (Jegou, 1992).

The cytoplasm of these cells are characterised as having a cytoskeleton, a large Golgi complex, smooth and rough endoplasmic reticulum, lipid inclusions, multivesticular bodies and heterophagic vacuoles.

Sertoli cells undergo morphological changes during maturation as well as during spermatogenesis (Jegou, 1992). Adult Sertoli cells display certain characteristics: the cytoplasm of these cells show a polar distribution, the organelles and inclusions are found in greater abundance in the basal trunk than in the apical portion which are not observed in immature cells (Verhoeven, 1999).

Russell *et al.* (1980) established that the Sertoli cell surface has filamentous materials deeply positioned through the endoplasmic reticulum. This characteristic is referred to as "ectoplasmic specialisations" which are seen at the level of the blood-testis barrier. Ectoplasmic specialisations appear to maintain a tight relationship with maturing spermatids

(elongated). These "ectoplasmic specialisations" may provide a mantle over the spermatid head, protecting the spermatid against trypsin degradation when releasing it into the lumen. It was further concluded by Russell *et al.* (1980) that "ectoplasmic specialisations" first come into association with a pachytene spermatocytes. These specialisations are found on both the apical and basolateral membranes of the Sertoli cell.

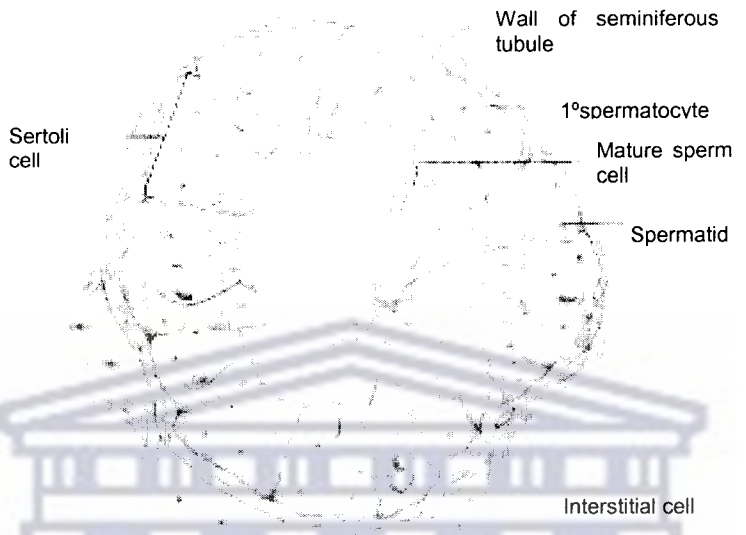

Figure 1.4. Cross section of the seminiferous tubule indicating the different cell types. Taken from Rhoades and Pflanzer (1996)

1.2.2 The Blood-Testis Barrier

Blood contains antigens and antibodies, which kill developing germ cells. For this reason an important physiological barrier exists which maintains a separation of blood plasma and the microenvironment of the Sertoli cell.

Studies in the rat have revealed that the blood testis barrier is established at approximately 15 days of age. The operation of the blood-testis barrier coincides with the initiation of spermatogenesis (Jegou, 1992). At 15 days after birth, junctional complexes begin to form between Sertoli cells near the base of the epithelium (Jegou, 1992).

The development of these tight junctional complexes forms the major component of the blood-testis barrier (Verhoeven, 1999). The junctional complexes located in the basal portion of the Sertoli cells divide the seminiferous epithelium into two compartments namely, the basal and the adluminal compartment. The basal compartment contains the primary spermatocytes and spermatogonia whereas the adluminal compartment contains the advanced spermatocytes and the spermatids (Setchell, 1979).

In all studied species, especially in primates, the blood-testis barrier has been shown specifically to reside in the specialised junctional complexes between the Sertoli cells (Jegou, 1992). In the rat and most rodents, however, the blood-testis barrier has been shown to reside partially in the peri-tubular layer of the myoid cells of the seminiferous tubules (Thibodeau and Patton, 1996), as well as the vicinity of the tight junctions between adjacent Sertoli cells. The importance of these tight junctions is emphasized in genetically modified mice, where they are absent, rendering the mice sterile (Gow *et al.*, 1999).

Substances from the extra-tubular compartment have direct access to the basal portion of the Sertoli cells. The inter-Sertoli cell specialised junctions, however, prevent direct access to the adluminal compartment. It has been ascertained that these tight junctions are stable and resistant to perfusion of hyperosmotic solutions, which are known to compromise other junctions (Jegou, 1992).

Significant differences between the fluids inside the seminiferous tubule and the blood plasma support the concept of the blood-testis barrier (Tuck *et al.*, 1970). Sertoli cells produce a unique microenvironment believed to be essential for germ cell differentiation.

Sertoli cell proteins regulate this microenvironment. The most prominent of these proteins are androgen-binding protein (ABP), transferrin, plasminogen activator and inhibin (Onoda *et al.*, 1990) as well as protease inhibitors, e.g. $\propto_2$-Macroglobulin (Cheng *et al.*, 1990). The luminal fluid of the seminiferous tubule contains a higher concentration in potassium and chloride (Levine and Marsh, 1971) and significantly lower concentrations of sodium, calcium, magnesium and bicarbonate phosphate than blood plasma (Tuck *et al.*, 1970). Furthermore, it has been shown with the use of tracer elements that wide variations exist in the rate at which various markers pass from the blood plasma into the testicular fluids. This ranges from instantaneous equilibration to virtually complete exclusion (Setchell, 1979) where certain tracer elements are inhibited from entering the adluminal compartment.

In order for the primary spermatocytes to migrate toward the adluminal compartment, a co-ordinated breakdown and reconstitution of the tight junctions must occur (Jegou, 1992). This happens as the proliferating and developing germ cells undergo incomplete cytokinesis, producing large colonies of cells that move in a co-ordinated fashion. This means that there is probably signalling between Sertoli cells and germ cells via the tight junctions (Verhoeven, 1999).

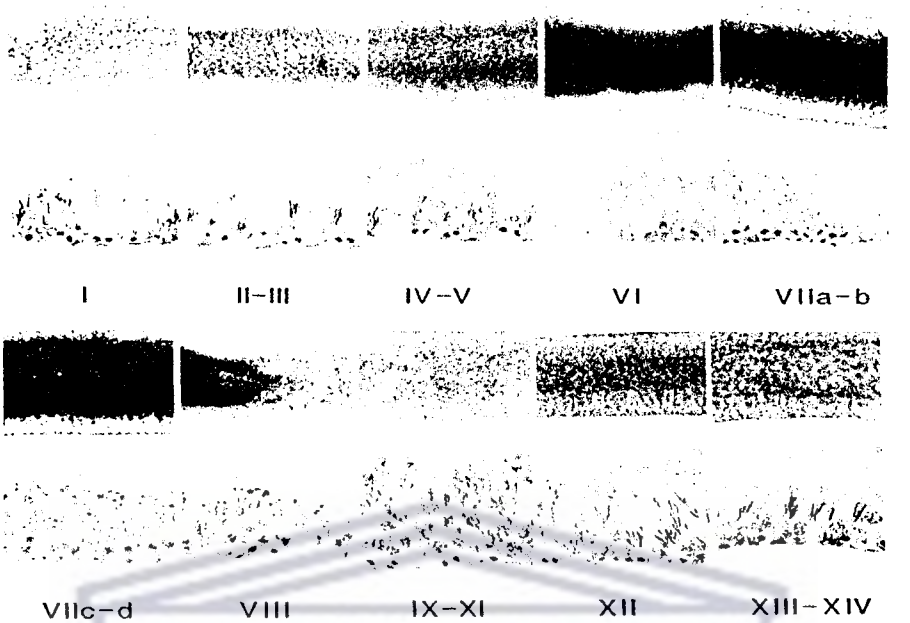

Figure 1.5. Transillumination pattern of freshly isolated rat seminiferous tubules related to the stage of the cycle of the seminiferous tubule. The roman numerals indicate the stages of the cycle as defined by the fixed stain sections along the lower portion of the figure. These are correlated with the transillumination of the unfixed tubules in the upper portion of the figure. Adapted from Parvinen and Ruokonen (1982).

This has been endorsed by the transilluminating experiments performed by Parvinen *et al.* (1980), which have revealed that the Sertoli cell undergoes cyclic functional changes during the progression of the spermatogenic cycle. Several stages have been identified within a spermatogenic cycle: 14 such associations have been identified in the rat and 6 in the human (Parvinen and Ruokonen, 1982).

Findings show that Sertoli cells in the seminiferous tubule of the rat are also electrically coupled through the existence of intercellular ionic communication between Sertoli cells (Eusebi *et al.* 1983). Secondary messenger molecules could be transferred via the low resistance pathways between epithelium cells to co-ordinate cellular activity and possibly the blood-testis barrier.

Thus major roles have been assigned to the blood-testis barrier. Promotion of the cohesion and cytoarchitecture of the epithelium, the constitution of a privileged microenvironment

required for the completion of spermatogenesis, prevention of contact of surface antigens with the spermatids and the immune system (Waites and Gladwell, 1982) constitute a few known ones. The BTB may also act to exclude potentially toxic substances from the seminiferous tubules (Setchell, 1979).

The most important function of the blood-testis barrier, however, must be in the creation of conditions favourable for meiosis (Setchell, 1979), allowing for the proper differentiation of spermatocytes into spermatids and spermatozoa.

These deductions have led to research involving exposure of a simulated BTB to products thought to be of contraceptive nature. Certain xenobiotics and drugs have been found to compromise the development of the BTB, and in so doing arrest the process of spermatogenesis. Sertoli cells have been established to possess a high sensitivity to the compound cadmium chloride (Janecki *et al.*, 1990) and that this chemical leads to a compromised blood-testis barrier, causing a drop in the secretion of certain proteins, Sertoli cell numbers and Sertoli cell viability.

It is this concept that serves as one of the motivations behind this project.

1.3 Secretory Function of the Sertoli cells

Sertoli cells have a very important secretory function. These cells produce the seminiferous tubule fluid, which provides the germ cells with the necessary nutrition (Tortora and Anagnostakos, 1987). This fluid is concentrated with proteins and also maintains a unique microenvironment essential for the survival and characteristics of the Sertoli cell, in that it contains multiple serum proteins.

Sertoli cell products have been used as probes to study the interactions of the Sertoli cells with their multiple germ cell types in the basal and adluminal compartments of the seminiferous tubules. It is assumed that these products may be useful in the establishment of interactions between germ cells and their nursing cells in the future.

With the use of techniques such as micropuncture (Tuck *et al.*, 1970), it was found that testicular fluid is produced by the seminiferous tubules and distal testicular structures.

A function of this secreted fluid is to transport chemical substances that possibly are involved in cell-cell communications between Sertoli cells and germ cells (Berne and Levy, 1993). This occurs from the basal portion of the seminiferous tubule epithelium. The fluid may also be responsible for the transport of the newly released sperm from the seminiferous tubule to the rete testis (Jegou, 1992).

Evidence observed in busulphan experiments support the theory that the Sertoli cell is the origin of this fluid. Busulphan is a chemical that destroys germ cells when doses of this chemical are administered peritoneally in young rats aged approximately 4 to 6 weeks. Brinster and Zimmerman (1994) demonstrated that young rats treated with busulphan still produced testicular fluid.

The tight junctions described earlier may be involved in the pumping of potassium and bicarbonate ions which are transported into the canaliculi making their content hypertonic (Jegou, 1992). This hypertonicity produces a water flux at the basal membrane of the Sertoli cell through a Na^+-K^+-ATPase exchange pump of the seminiferous tubule (Waites and Gladwell, 1982).

At about the same time as fluid production begins, the mitotic divisions of the Sertoli cell cease and they differentiate rapidly (Jegou, 1992). This fluid production has been found to be under the influence of hormones.

Apart from the secreted fluid, the Sertoli cell also secretes several proteins. The first protein to be discovered was the androgen-binding protein (ABP) in the rat (Bardin *et al.*, 1994) and has provided a prototype for other Sertoli cell secretory proteins. This glycoprotein is secreted in the Sertoli cells as a response to hormone action on the seminiferous tubules. ABP binds certain hormones with high affinity (Berne and Levy, 1993), thus regulating their availability to germ cells in the seminiferous tubular fluid and to sperm within the epididymis.

Germ cells are noted to have specific binding sites for ABP. The germ cells internalise ABP via a receptor-related endocytotic pathway. It can thus be assumed that ABP may be required for the development of germ cells (Gerard *et al.*,1994).

Other secreted proteins that have been identified are inhibin, plasminogen activator, as well as other small peptides. Inhibin, a glycoprotein hormone is produced by the Sertoli cells in

response to hormone secretion (Raivio *et al.*, 2000). In addition to suppressing hormone secretion, inhibin may also have effects on the neighbouring cells, namely the Leydig cells and germ cells (Berne and Levy, 1993). The Sertoli cell has also been observed to produce an activin subunit and follistatin along with inhibin, demonstrating their potential to regulate germ cell as well as their own development (Meehan *et al.*, 2000).

Plasminogen activator has been identified as having a role in the release of preleptone spermatocytes from the basement membrane at the onset of meiosis (Toppari *et al.*, 1986). The secretion of this protein was found to be stimulated by FSH.

Other small peptides, like metal-binding proteins secreted by the Sertoli cells, have been identified in low concentrations and these peptides may possibly be involved in local cell-cell communications within the seminiferous tubule.

In experiments by Cheng *et al.* (1990) it was found that the Sertoli cell synthesises and secretes a protease through degenerating late spermatids. These degenerating spermatids release protease, which can damage the seminiferous tubules and the rest of the genital tract if they are not inactivated (Cheng *et al.*, 1990). The mechanism by which proteins are expelled is not known but it is proposed that the protease form covalent bonds with α_2-macroglobulin. The resulting complex is probably cleared from circulation by a receptor-mediated mechanism. Apart from acting as protection, redirecting α_2-macroglobulin is known to stimulate the growth of some cells *in vitro*. This could occur as a result of bonds forming between factors and the molecule (Cheng *et al.*, 1990).

The ability of adult rat Sertoli cells to metabolise certain androgens indicates the presence of certain enzymes in the Sertoli cell. Procysterone is metabolised into testosterone and dihydrotestosterone (DHT) in the Sertoli cell. Testosterone, in turn, is converted to DHT or to androstenone. This takes place in the presence of the enzymes 5α-reductase, steroid oxidoreducers and lyase (Tcholakian and Steinberger, 1980).

In addition to protein and peptides, Sertoli cells have a marked capacity for synthesising and metabolising many steroids (Jegou, 1992). The nuclear and cystol androgen receptors found in the Sertoli cell bind to Sertoli cell chromatin and secrete products in response to the stimulation of these receptors (Parvinen and Ruokonen, 1982).

Sertoli cell products are secreted bi-directionally as a result of the polarised morphology of the cell. Products are secreted via the apex of the cell into the tubule fluid, and via the base of the Sertoli cell into the interstitial fluid and then into the blood (Jegou, 1992).

Developing germ cells in the rat seminiferous epithelium form 14 associations or stages of constant and predictable composition. It is thus possible to study the function of Sertoli cells as it relates to various stages. There are stage dependent differences in the secretion of ABP, and stages at which the maximum ABP secretion is noted (Parvinen and Ruokonen, 1982).

Experiments by Gerard *et al.* (1994) showed that maximum nuclear labelling occurred in stages VII and VIII of the seminiferous cycle under the influence of testosterone. This demonstrates that internalisation is dependent, to a large extent on the concentration of testosterone in the seminiferous tubule.

1.4 Hormonal regulation of Sertoli cell function

The major humoral regulators of the seminiferous epithelium *in vivo* are testosterone and FSH. Sertoli cells control the internal environment of the testes under the influence of these main as well as other humoral factors.

Follicle stimulating hormone (FSH), a pituitary glycoprotein is essential for normal sexual development and reproduction function (Davis and Bordy, 1980). It controls a number of morphological and biochemical processes e.g. cell shape, protein secretion and estradiol production (D'Agnostino *et al.*, 1992).

It has been suggested that many of the hormonal effects on spermatogenesis are executed via the Sertoli cells (Jegou, 1992). A host of biochemical events is triggered within the cell as a result of FSH action. These events include the activities of odenglyl systose, a decreased actuary of phosphodiesterase. This process results in an enhanced steady-state level of cAMP. Increased cAMP levels bring about an activation of cAMP-dependent protein kinase. This leads to phosphorylation in the Sertoli cell. Protein like inhibin B is produced by Sertoli cells in response to FSH (Raivio *et al.* 2000).

Regulation of Sertoli cell function requires the action of two pituitary hormones: FSH and LH (luitenising hormone) as suggested by Verhoeven (1999). FSH acts directly on the seminiferous tubules, binding to specific receptors on the Sertoli cell (Jegou, 1992) whereas LH acts on the Leydig cells to guarantee high local levels of androgens.

There is convincing evidence that the Sertoli cells are the primary targets for FSH in the testis, the FSH receptors being exclusive to these cells (Parvinen *et al.*, 1980).

The FSH action first involves an interaction with its specific membrane receptor, this complex subsequently becomes internalised and metabolised (Jungwirth *et al.*, 1997).

The reaction is required for stimulation of adenylate cyclase which catalyses the production of cyclic adenosine 3'-5' monophosphate (cAMP) and activating protein kinase (Parvinen *et al.*, 1980). FSH stimulation of Sertoli cell metabolism is correlated with intracellular cAMP levels (Davis and Bordy, 1980), confirming the assumption made by Dorrington and Fritz (1974) that the Sertoli cell responds to FSH with an increase in cAMP levels.

A variety of transcription and translational events have been attributed to FSH (Parvinen *et al.*, 1980). FSH stimulates the Sertoli cells to produce a signalling molecule known as Kit-ligand, which promotes DNA synthesis in spermatogonia (Verhoeven, 1999).

FSH plays a major role in Sertoli cell function. This hormone is responsible for initiating an irreversible developmental event association with the initiation of spermatogenesis (Davis and Bordy, 1980).

Specifically, FSH has exhibited the induction of protein synthesis (D'Agnostino *et al.*, 1992). Gonadotropin drive to the testis is the most important factor stimulating inhibin secretion (Raivio *et al*, 2000) as well as other proteins, peptides and steroids essential for Sertoli cell function viz. ABP (Berne and Levy, 1993), plasminogen activator (Onoda *et al.*, 1990) and other important compounds.

FSH has been shown to induce hyperpolarisation of the resting membrane potential of the Sertoli cell, and regulates the tight junctional complex between Sertoli cells (D'Agnostino *et al.*, 1992). The complex is then regulated by a calcium conductance, which is also regulated by FSH (Joffre *et al.*, 1988).

The interstitial or Leydig cells, which contain LH receptor proteins, are responsible for secreting testosterone under stimulation by LH, acting on the Sertoli cells in a paracrine fashion (Tortora and Anagnostakos, 1987). Testosterone has been shown to exhibit protein changes in the size testes. It was therefore concluded that testosterone is responsible for major changes in mature Sertoli cells (Mills, 1990).

The testosterone diffuses across the basement membrane of the seminiferous tubules and into the Sertoli cells. Testosterone is converted by the enzyme 5-∝-reductase into dihydrotestosterone and estradiol, the active metabolites that influence spermatogenesis (Berne and Levy, 1993).

The completion of spermatogenesis is dependent on testicular androgens that exert their effects on the germ cells exclusively through the Sertoli cells (Parvinen and Ruokonen, 1982).

Experiments performed by Parvinen and Ruokonen (1982) have further demonstrated that FSH exhibits differential binding and action depending on the seminiferous epithelial stages. It has further been determined that acute withdrawal of gonadotropins leads to stage specific loss of germ cells at the hormone dependent stages of the seminiferous epithelium. These are at stages VII and VIII in the rat (Lue *et al.*, 2000). These stages have been utilised as a model for studying Sertoli cell/germ cell interactions.

It has also been shown by Van Damme *et al.*, (1980) that the Sertoli cell aromatises androgens under the influence of FSH. Other pituitary hormones play a role in the regulation of Sertoli cells. Growth hormone, e.g. has been proposed to stimulate local production of insulin-like growth factors by Sertoli cells (Berne and Levy, 1993).

It should be noted that neither FSH nor LH appear to act directly on the germ cells, i.e. they act exclusively on the intervening Sertoli and Leydig cells respectively (Verhoeven, 1999).

It can therefore be concluded that the Sertoli cell is non-functional without the secretion of gonadotropins and thus spermatogenesis is non-existent or incomplete without gonadotropin presence (Bardin *et al.*, 1994).

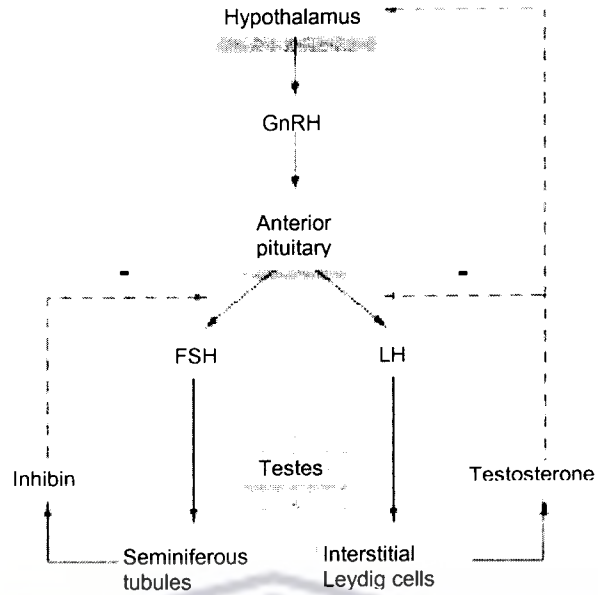

Figure 1.6. Hormonal regulation of the seminiferous tubules.

1.5 The Electrical Potential of the cell

All cells have an electrical potential. This is due to the cell cytoplasm being more negative in comparison to the extracellular environment (Berne and Levy, 2000). This state of electrical potential is known as the resting membrane potential.

Cell membranes are practically impermeable to intracellular proteins but are moderately permeable to Na^+ and freely permeable to K^+ and Cl^-. These ions cross the membrane via chemically-gated channels and voltage-gated channels (Ganong, 1983).

The electrical potential difference across the plasma membrane results in a prevention of ions within the cell passively diffusing out. The electrical potential difference influences the direction of diffusion of ions across the cell membrane (Fox, 1996). For example, Cl^- diffuses into the cell against an electrical gradient due to the cytoplasm being more electrically negative to the extracellular fluid as well as a chemical gradient, as the extracellular environment has a greater concentration of Cl^- ions.

Na^+, for example, is actively pumped out of the cell via the Na^+ K^+ ATPase pump and K^+ in return is pumped into the cell. Because Na^+ is found in higher concentration in the

extracellular fluid, it is pumped out against a large electrochemical gradient (Berne and Levy, 2000).

The magnitude of the membrane potential at any given time depends on the distribution of Na^+ K^+ and Cl^- and the permeability of the membrane to these ions (Ganong, 1983).

Ion currents also pass through separate Na^+ and K^+ channels in the plasma membrane. These channels work via activation gates activated by a response to a receptor protein on the membrane coming into contact with specific ions or substances. The geometric shape of the channel pore determines which substances are allowed through the pore.

Because of its electrogenic nature (carries a net transfer of positive charge out of the cell) the Na^+ K^+ ATPase pump contributes directly to the resting membrane of the Sertoli cell and possibly the function of this cell.

Electrical coupling between cells is known to be a function of tissue maturation and of hormone-dependent development processes. Intercellular electrical coupling has been noted as one of the possible mechanisms of interaction between cells in the seminiferous epithelium (Eusebi *et al.*, 1983).

1.6 Electrophysiology of the seminiferous tubules and the Sertoli cell

1.6.1 Introduction

In 1970 Tuck and colleagues used microelectrodes to measure the potential difference across seminiferous tubules for the first time. This study showed that the lumen was negative with respect to the interstitium. This finding was concluded by Cuthbert and Wong (1975), who, with the use of microelectrodes recorded the intracellular potentials of the cells in the seminiferous tubules. Their studies further showed a varied potential from –8mV and –50mV with a mean of -28.2mV. There was one pitfall of this study: they did not distinguish which cells of the seminiferous tubule were impaled i.e whether they had impaled the surrounding myoid cells, germ cells or Sertoli cells.

This work led to the study of the electrophysiological properties of cultured Sertoli cells grown on plastic surfaces. Cells grown on plastic showed that they grew to resemble flat, squamos-like cells, unlike cells cultured on a suitable matrix, which grew into columnar-like cells (Hadley *et al.*, 1985). Subsequently, techniques for growing confluent Sertoli cell monolayers were developed (Janecki *et al.*, 1990) and attempts were made to mimic the *in vivo* environment of these cells.

Figure 1.7 Light micrographs (x780) of Sertoli cells grown in serum-free media in the presence of various substrata. Top: Cells on plastic appear as low squamos cells. Bottom: Sertoli cells grown on a reconstituted basement membrane. Sertoli cell nuclei (S) and apical surface (A) are indicated. Taken from Bardin et al., (1994)

1.6.2 Electrophysiological Investigations

Different electrophysiological techniques have been developed to understand the ion transport characteristics of cells. The technique of intracellular microelectrode recordings makes use of microelectrodes with tip diameters minute enough to penetrate the seminiferous tubule membrane as well as the Sertoli cell membrane causing as little damage as possible.

Another technique known as "Patch-Clamping" makes use of the formation of an electrical seal around a micropipette with a high tip resistance. The high resistance of the seal ensures

that most currents originating in a small patch of membrane flow into the pipette and thus the circuit measuring apparatus (Hamill *et al.*, 1981). This makes it possible to observe the currents through single ionic channels.

Different compounds, known to block certain ion channels, can be added and the potential differences can be measured and compared to the channel/pump population of the membrane. This could indicate the permeability of the membranes and the sensitivity of the cell to different compounds.

Micropuncture studies were used to study some of the electrochemical aspects of fluid and electrolyte transport in seminiferous tubules. These studies revealed that seminiferous tubules contain a fluid that is slightly hypertonic to plasma, having a higher potassium and chloride ion concentration than plasma (Levine and Marsh, 1971; Tuck *et al.*, 1970).

In vitro experiments using microelectrodes have been used to record intracellular potentials from cells of the seminiferous tubules of rats. The average resting membrane potential of rat seminiferous tubules was recorded as being -28mV±0.3mV at 33°C (Cuthbert and Wong, 1975; Jungwirth *et al.*, 1997).

The use of this electrophysiological technique has been used in investigating the effects of hormones on the membrane of Sertoli cells *in vitro*. This has led to breakthroughs in understanding functions of the Sertoli cell and the response of Sertoli cells to hormonal regulation and other compounds. Because of the relatively few electrophysiological studies on Sertoli cells, only the main findings will be summarised in the section below.

The effect of FSH on the electrical properties of cultured TM4 Sertoli cells was investigated using the technique described above. The potential of the cells depolarised by +15.3mV, but this depolarisation was completely abolished in a chloride free solution. It was concluded by Jungwirth *et al.* (1997) that FSH conducts a chloride conductance in cultured TM4 Sertoli cells.

In addition to the above findings, it was established that the junctional complex between Sertoli cells is regulated by calcium ions (D'Agnostino *et al.*, 1992). FSH was found to induce hyperpolarisation of the cell.

Electrophysiological experiments performed by Ko *et al.* (1998) have demonstrated that the seminiferous epithelium possess great resistance to pharmacological agents. The use of the above method of research makes use of compounds whose effects on certain ions are known.

It has been established that verapamil, an inhibitor of calcium channels, can be used to block the hyperpolarisation of the Sertoli cell caused by FSH (Jungwirth *et al.*, 1997) and that this effect was dependant on the chloride concentration of the bath Ringers.

It has been demonstrated that electrogenic ion transport by ATP may be part of a complex mechanism regulating fluid secretion by the testis (Ko *et al.*, 1998).

Ouabain, a glycoside, was found to depolarise the membrane of the seminiferous tubule. It was further established that 2,4,-dinitropherol affected the membrane in the same way (Cuthbert and Wong, 1975).

Electrophysiological experiments by Lalavée and Joffré (1999) using the whole cell patch-clamp technique have shown that internal Ca^{2+} activates an outward rectifying current. This current reverses to Cl^- equilibrium potential and is inhibited by Cl^- channel blockers. While remaining insensitive to TEA, an inhibitor of Ca^{2+}-dependent K^+ current. It has been assumed that Ca^{2+} interacts directly with Cl^- channels.

The following compounds have been shown to affect the membrane of the Sertoli cell in the following way. Amiloride blocks epithelial sodium channels, acetazolamide and 6-ethoxyzolamide inhibits carbonic anhydrase, chlorothiazide inhibits the Na^+Cl^- cotransporter, H_2DIDS inhibits the Na^+ HCO_3^- cotransporter and bafilomycin inhibits vacuolar H^+ ATPase (Ko *et al.*, 1998).

Joffre *et al.* (1988) established with the use of intracellular microelectrode recordings that FSH caused the hyperpolarisation of cultured Sertoli cells and an increase in Na/Ca influx and K^+ efflux. This effect was found to be reversible and mediated by cAMP.

Using the whole-cell patch clamp, Joffre *et al.* (1988) also established that (HCG) human chronionic gonadotropin stimulation involved the blockade of a potassium current and the activation of a chloride current through the increase of intracellular calcium in Sertoli cells.

A study by Gladwell (1977) indicated that the seminiferous tubule potential difference is maintained by a temperature sensitive, cellular mechanism. The exposure of the testes to severe low body temperatures depolarised the seminiferous tubules, and in so doing, increased their permeability.

Of importance is to note that all the above experiments on mammalian tissues were performed on cultured primary Sertoli cells and that no attempt has been made to firstly establish the growth patterns on other immortalised cell lines, e.g. TM4 Sertoli cells, in comparison to primary cultures. Furthermore, no microelectrode studies have been performed on cell lines grown to mimic the *in vivo* environment and that the electrophysiological properties may differ between these different cell cultures.

An intracellular recording study performed on the Malpighian tubules of the locust performed by Baldrick *et al.* (1988) was the first study to measure the potential difference across both the apical and basolateral membranes of a cell using a bicameral system. The experimental design of this study would later lay the foundation for further studies on cells grown in bicameral systems. This study also introduced the use of ionic transport inhibitors to characterise different cotransporters in the membranes of cells.

1.7 Conclusion

A major advance in the study of Sertoli cells has been the development of procedures for *in vitro* studies. This has led to the identification of factors required for their growth and differentiation, the development of junctional complexes and thus the BTB. Cell culture procedures have also allowed for the *in vivo* epithelia to be mimicked, allowing scientists to generate an understanding of the importance of the BTB. It is this understanding of the importance of Sertoli cells that has motivated this study. It is believed that a compromising of the BTB can lead to arrested development of germ cells. This project aims to test whether plant extracts are capable of achieving this and leading to a possible contraceptive being developed.

It is hypothesized that a breakdown in the BTB results from a possible change in the transepithelial resistance across the seminiferous tubule brought about by substances, which prevent the tight junctions from forming between adjacent Sertoli cells. It is further

hypothesized that the breakdown of this transepithelial resistance leads to a compromise of the membrane ionic transport mechanisms.

This chapter was designed to give the reader a background to the subject matter presented in this thesis. The chapters that follow include the studies performed on the TM4 Sertoli cell line.

Chapter 2 reports on the anti-fertility and toxicological screening of the medicinal plant extracts used in the electrophysiological experiments. It serves as the motivation behind using these extracts in the electrophysiological studies. This chapter has been written up in the form of an article for The Journal of Andrology.

Chapter 3 gives a detailed description into the cell culturing, apparatus, solutions, drugs and electrophysiological techniques used in this study. The importance of the above protocols is discussed here.

Chapter 4 reports on the electrophysiological experiments performed on the cell cultures. This is followed by chapter 5, which discusses the outcomes of the experiments and attempts to put together a feasible conclusion for the data received.

All the references used in this study follows chapter 5, along with the appendices.

CHAPTER 2

THE EFFECT OF INDIGENOUS PLANT MEDICINE EXTRACTS ON MALE REPRODUCTIVE HEALTH AND METABOLISM

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Abstract

In male albino mice, the ethanol extracts of *Olea europaea* and *Olea exasperata* were compared with oleanolic acid as potential contraceptive agents. A 45 day *in vivo* study investigating these extracts revealed a total inhibition of fertility in animals treated with oleanolic acid, a 66% inhibition in *Olea europaea* treated animals and 33% inhibition of fertility in animals treated with the extract of *Olea exasperata*. Further tissue histology of the testes revealed that the testes sections showed a breakdown in spermatogenesis. Haematology investigation revealed animals that underwent oleanolic acid treatment showed no significant differences to that of the controls, remaining in the normal range for blood indices. Mean corpuscular haemoglobin volume for red cells decreased in the group treated with *Olea europaea* ($P<0.02$) and percentage neutrophils ($P<0.03$), lymphocytes ($P<0.01$) and basophil ($P<0.05$) showed significant changes in these mice, lying beyond the normality range for these indices. These blood cell parameters may mean that the treated animals are susceptible to disease, but is inconclusive at this point. *Olea exasperata* treatment showed no negative effect on blood parameters, but little contraceptive effect. This study demonstrates that plant extracts may well be utilized for

contraceptive purposes. The administration of these extracts needs to be monitored over a larger population for a longer period before we could conclusively claim that they could be utilized as reversible and safe contraceptive agents.

2.1. Introduction

The use of medicinal plants as reproductive system remedies has been an age-old practice. Chinese scientists in particular have made extensive use of their rich ethnomedical knowledge and have capitalised on the rich flora of China and the experience in the field of phytomedicine (Phillipson, 2001).

Ayurvedic medicine from the Indian sub-continent is suggesting important leads for researchers in this field. Ancient Indian literature abounds with information on large numbers of plants reputed to have sterilizing, contraceptive and abortifacient properties. Scholars of Ayurveda have also mentioned several plants in their Ayurvedic treatises. Ayurvedic physicians all over India, who claim their effectiveness but are unable or unwilling to produce data, are still using a number of these preparations (Hoffmann, 2002).

This campaign has led to the discovery of natural anti-implantation agents as well as a male contraceptive (Kong *et al.*, 1986). It is this male contraceptive that is investigated in this study as well as crude extracts from plants known to possess this compound.

Oleanolic acid (*3B-hydroxy-olea-12-en-28-oic acid*) is a triterpenoid compound existing in a large number of plants (Liu, 1995). Isolated from these many plants families, this compound was found to exhibit definite antifertility in mice (Kong *et al.*, 1986) and in the rat model (Rajasekaran *et al.*, 1988). This compound has been shown to be relatively non-toxic with no noted side effects over long periods of time (Xu, 1985).

Amongst the many plants that have been identified as being producers of oleanolic acid are those of the family of Oleaceae (Garcia- Garnardos *et al.*, 1998). It is from this family

that the plant species for this study have been selected to establish the claimed antifertility effect of these medicinal plants. Oleanolic acid has been shown to be an active component in producing hepatoprotection effects (Liu, 1995). Already, this compound is being marketed as an oral drug to treat human liver diseases in China, including hepatitis (Liu, 1995).

Many triterpenoids possess an anti-inflammatory effective property. Oleanolic acid has been shown to be responsible for suppressing adjuvant-induced arthritis in rats and mice (Singh *et al.*, 1992).

Tumor initiation and promotion are inhibited by oleanolic acid to various degrees. The most notable effect of this triterpenoid is anti-tumor promotion (Liu, 1995). The mechanisms by which oleanolic acid suppresses tumor promotion is not known, but might be a result of an inhibition of inflammation produced by the tumor or a promoted suppression of oncogene expression (Tokuda *et al.*, 1986).

In Japan, the use of oleanolic acid and ursolic acid has been recommended for skin cancer (Muto *et al.*, 1990; Granger and Scott, 1998). It has been found that oleanolic acid exerts anti-hyperlipidemic effects (Liu, 1995). OA decreases the elevated blood cholesterol levels and prevents lipid precipitation in blood vessels and major organs of experimental hyperlipidemic rabbits (Liu, 1995).

OA extracted from *Eugenia jambolana* was shown to have antifertility effects in male rats, without affecting their libido. This was established by Rajasekaran, *et al.* (1988), when male rats were given a diet supplemented with OA. Vaginal smears of the females gave evidence that mating took place in all cases of treated rats. However, only 10% of the female rats conceived. It was further established that this compound and others have been identified as inhibitors of testosterone 5 α-reductase and have been shown to have anti- male hormone activities (Rajasekaran *et al.*, 1988).

Some other activities that have been attributed to OA are the anti-ulcer effects and the antimicrobial activities that OA exhibits (Liu, 1995). Apart from all the above uses for OA, it has been proven that OA is relatively non-toxic, whether applied over a short period of treatment or over an extensive period of time. Because of this, OA has been patented in Japan as an additive to health drinks and has also been marketed in China for liver disorders. Recently, a procedure was established by Garcia-Granados *et al.* (1998), which effectively yielded over 5g of OA from 1kg of *Oleaceae* leaves. It is this family of plants that serves as the subject for this study.

It is the aim of this study to determine the effects of crude extracts derived from these plants in the impairment of fertility in male albino mice. Furthermore, this study also aims to assess the safety of these extracts by evaluating a variety of blood indices and the cellular integrity of selected tissues.

2.2 Materials and Method

2.2.1 Plant Material

The leaves of *Olea europaea* and *Olea exasperata* were collected from the Western Cape region of South Africa. These were carried out under the supervision of F. Weitz, a botanist of the University of the Western Cape. Leaves showing no fungal and bacterial infection were collected, as infection is known to alter plant metabolism, which in turn could lead to unexpected products being produced in a high concentration (Harborne, 1983).

Since plant species was collected far from a laboratory, it was not possible to use fresh plant tissues for phytochemical extraction. Therefore, approximately 100g of leaves were bottled in methanol on site, while the rest of the leaves were placed in paper bags to ensure that the plant material would dry naturally and be kept in a good condition for later use. A few leaf specimens were also immersed in formalin, which preserved them

for herbarium studies. The bagged plant material was dried at 40°C in an incubator, preventing chemical transitions from occurring.

2.2.2 Extraction procedure

For the extraction of chemical compounds present in the plant tissue, it was necessary to use phytochemical methods. In this case, a crude extract was created to screen for the possible activity of oleanolic acid (OA), a triterpenoid compound known to be contraceptive in males (Liu, 1995). This would be used in future experiments to compare the crude extracts to purified oleanolic acid. OA is a triterpene derived from a family of terpenoids, biogenetic derivates of the isoprene molecule (Harborne, 1983). It can be extracted from plant tissues with light petroleum ether, ether or chloroform and can be separated by chromatography on silica gel (Rajasekaran *et al.*, 1988).

For extraction, the collected plant material was first destroyed to prevent any enzymic oxidation or hydrolysis from occurring. This was done by macerating the leaves in a blender, immersing the macerated material in methanol and then filtering the solution. Once the tissue debris was colourless, it was assumed that all the compounds were extracted.

The extract was clarified under rotovaporation, concentrating the bulky solution down to small volumes at a temperature of 40°C. Once the methanol was removed via this process, the resulting solution, assumed to be water was freeze dried. Ethanol (10%) was used to suspend the extract in. This process theoretically yielded 0.5g OA from the 100g of macerated material.

For the *in vivo* antifertility investigation, an ethanol solution of crude extract calculated to theoretically contain 5mg/ml OA was made up. 0.2ml of this solution theoretically contained the equivalent of 30mg/kg mouse. This would be screened against the effect of OA 30mg/kg body weight, a concentration established to induce 100% sterility in rats and mice (Rajasekaran *et al.*, 1988).

2.2.3 Animals

Twenty-four adult NMRI outbred male albino mice of sexual maturity (SAIMR, Ltd), approximately 6 to 8 weeks old and weighing 34±40g (Table 2.1) were used in this investigation. They were maintained in the Department Animal House Facility with a 12/12-hour light schedule. The animals were fed with standard commercial diet (Epol. SA. Ltd) and water was allowed *ad libitum*. The animals were randomly separated into 4 groups of 6 each.

Group one served as control, receiving a daily dose of 10% ethanol solution. Group two received a solution of OA (30mg/kg animal) in 10% ethanol per day. Group three was treated with 10% ethanol extract of the leaves of *Olea europaea* (OU) made up in concentration containing 30mg/kg animal. Group 4 was treated with the extract from the leaves of *Olea exasperata* (OX) prepared as per OU. The crude extracts were administered in a dose calculated to contain 30mg/kg OA per animal. Treatment was administered intraperitoneally for 45 days.

2.2.4 Autopsy

After 45 days treatment, each of the 24 males were paired with a female of the same age for 24 hours. During this 24-hour window, the animals were monitored visually for the frequency of copulation. After this period, the male mice were sacrificed under anesthesia using chloroform/ ether (1:1), whereafter a blood sample was obtained immediately via cardiac puncture. The testes of these mice were removed immediately and immersed in Bouin's solution to preserve them for cellular assessments. After a further 25 days the females and all offspring, if any, were sacrificed.

2.2.5 Parameters

2.2.5.1 Body and testis weight

The body weights of the animals were recorded before and after treatment. At sacrifice, the testes were removed from all groups and compared with the control (table 2.1). This would serve as indication as to whether administration of the test substances had any effect on the diets of the animals or on the constitution of the testes.

2.2.5.2 In vivo Fertility Test

After treatment, all the males were paired with a female of sexual maturity. Visually monitoring copulation for 24 hours as well as observing the presence of a natural plugging mechanism in the vagina of mated mice confirmed success of mating. The females were monitored over a period of 25 days for conception. Litter size, if any, were recorded.

2.2.5.3 Haematological Screening

A blood sample from each male was collected after the 45 days treatment by way of cardiac puncture. Approximately 3ml of blood were collected per mouse and placed into EDTA vacuum containers. The samples were analysed on a Coulter MD 18 haematology analyser within 8 hours following collection. Total red blood cells, haemoglobin, haematocrit, mean corpuscular volume (MCV) and mean corpuscular haemoglobin (MCH) were determined as the parameters for red cells. These parameters indicate the ability of the red blood cells to transport oxygen in the blood. Red blood cell indices are also used to detect abormalities in size, denoted by the MCV. It also allows for the determination of erythrocyte haemoglobin concentration by the measuring of the MCH. Mean corpuscular haemoglobin concentration (MCHC) is the index for the proportion of haemoglobin measured per average red blood cell. A low MCH and MCHC indicates possible hypochromia (inadequate haemoglobin), while high values indicate

microcystosis (loss of red blood cell volume without a proportionate loss of haemoglobin (Matthews and Van Holde, 1992). Measuring the haemoglobin levels gives us an indication as to whether the red blood cells are capable of transporting gases. A lack of haemoglobin leads to anemia, which impairs systemic function and is a sign of disease. Assessing the haematocrit levels also monitors anemia, the minimum accepted level being 37% (Berne and Levy, 2000).

For white cell parameters, total white cells, neutrophils, lymphocytes, monocytes, basophils, eosinophils and platelet count were determined. The analysis of these parameters would serve as an indication as to whether the immune system was challenged in any way. White blood cells function collectively to combat foreign substances in the body. These cells are defensive cells that phagocytize material, detoxify poisons, produce antibodies, release chemical receptors and secrete enzymes (Fox, 1996). The higher the concentration of white blood cells in the blood, the greater the chance of the animals' immune system being compromised. A rise in the neutrophil levels is associated with inflammation, an increase in the eosinophil level is generally associated with allergic conditions and an increase in the basophils, monocytes and lymphocytes is generally associated with foreign antigens in the body (Sherwood, 2001). Platelet count gives a measurement of the self-healing capabilities in the animal's body. Following injury, chemicals in the platelets stimulate the contraction of the injured vessels and are capable of repairing damage to the vessels (Matthews and Van Holde, 1992).

2.2.5.5 Tissue histology

Upon sacrifice, the testes were removed from the animal and immersed in Bouin's solution for 48 hours. The Bouin's solution was drained and the preserved organs dehydrated in ascending grades of alcohol and xylene, whereafter it was suspended in wax for 24 hours. After this waxing period, the organs were embedded in wax blocks and frozen for 24 hours. The blocks were then cut into 4μm thick sections and mounted on slides. The slides then underwent a hydration process, embedded in xylene and then descending grades of ethanol before being immersed in water. They were then exposed to

haematoxillin stain for 15 minutes, rinsed and briefly immersed in Scott's tap water. Thereafter they were rinsed again and briefly placed in ethyl alcohol, rinsed and stained with eosin for 30 seconds and rinsed again. The slides were then dehydrated in ascending grades of alcohol, immersed in xylene and incubated for 2 hours. They were then fixed with Canada balsam and coverslips. Photos under light microscopy were then taken with a Winder M camera (model: 476079-9901).

2.2.6 Statistical analysis

Continous data was tested for normality using a normality plot in Medcalc. None of the data sets were considered to be normal, so non-parametric analysis of variance (Kruskal Wallis) was conducted for all data sets using Medcalc (version 4.16f – 1997). The parameters that demonstrated significant differences between treatments were analysed using Wilcoxon unmatched pairs analysis to establish which treatments were different from each other. Null hypothesis: there exists no difference between treatment A and treatment B.

2.3 *Results*

2.3.1 *Body and testis weights*

No changes were observed in the body weight of the treated animals. A testicular weight comparison also showed no significant decrease in comparison to the control (Table 2.1).

Table (2.1) Body and testis weights of mice following treatment with ethanol extracts of Olea europaea, Olea exasperata and oleanolic acid.

Group	Initial body weight	Final body weight	Testis weight
Control	33.50±1.51g	33.00±1.70	0.15±0.03
Oleanolic acid	33.67±1.72g	33.82±1.45	0.15±0.01
Olea europaea	35.67±1.89g	34.58±1.18	0.15±0.02
Olea exasperata	34.33±2.03g	33.50±2.24	0.15±0.01

2.3.2 *In vivo Antifertility test*

An effect was observed in the treated males paired with the females. Libido of the treated males was unaffected, denoted by the "plugging" of the vagina upon copulation. This plugging results from the continued secretion of mucus from the cervix due to excessive exposure to sperm. This secreted mucus then solidifies to form a plug (Sherwood, 2001).

There was a 0% fertility rate in OA treated animals, a 33% fertility rate in OU treated animals and 66% fertility rate in OX treated animals in respect to the number of females that conceived. The results of this test are best annotated in table 2.2 below.

Table (2.2) Effect of ethanol extracts of Olea europaea, Olea exasperata and oleanolic acid on fertility of adult male mice.

Group	No. of mated males/females	No. of females delivered	No. of offspring Produced	Percent Fertility
Control	6/6	6	56	100
Oleanolic acid	6/6	0	0	0
Olea europaea	6/6	2	11	33
Olea exasperata	6/6	4	23	66

2.3.3 Haematological Investigations

Using the Kruskal Wallis analysis of variance, it was established that 7 of the 19 parameters measured exhibited significant differences between treatments. Wilcoxon analysis showed significant differences in these 7 parameters.

The animals treated with OA and the crude extracts OU and OX did not reveal many significant differences in comparison to the control. Total red cell counts remained similar, except for the mean cell haemoglobin ($P<0.04$), mean cell volume ($P<0.03$), which underwent a decrease in treated groups and the RDW ($P<0.01$), which differed significant from the control. For the white blood cell parameters, the percentage neutrophils ($P<0.03$), neutrophil Abs count ($P<0.05$), monocyte Abs count ($P<0.02$) and percentage lymphocytes ($P<0.01$) showed significant changes in comparison to the controls.

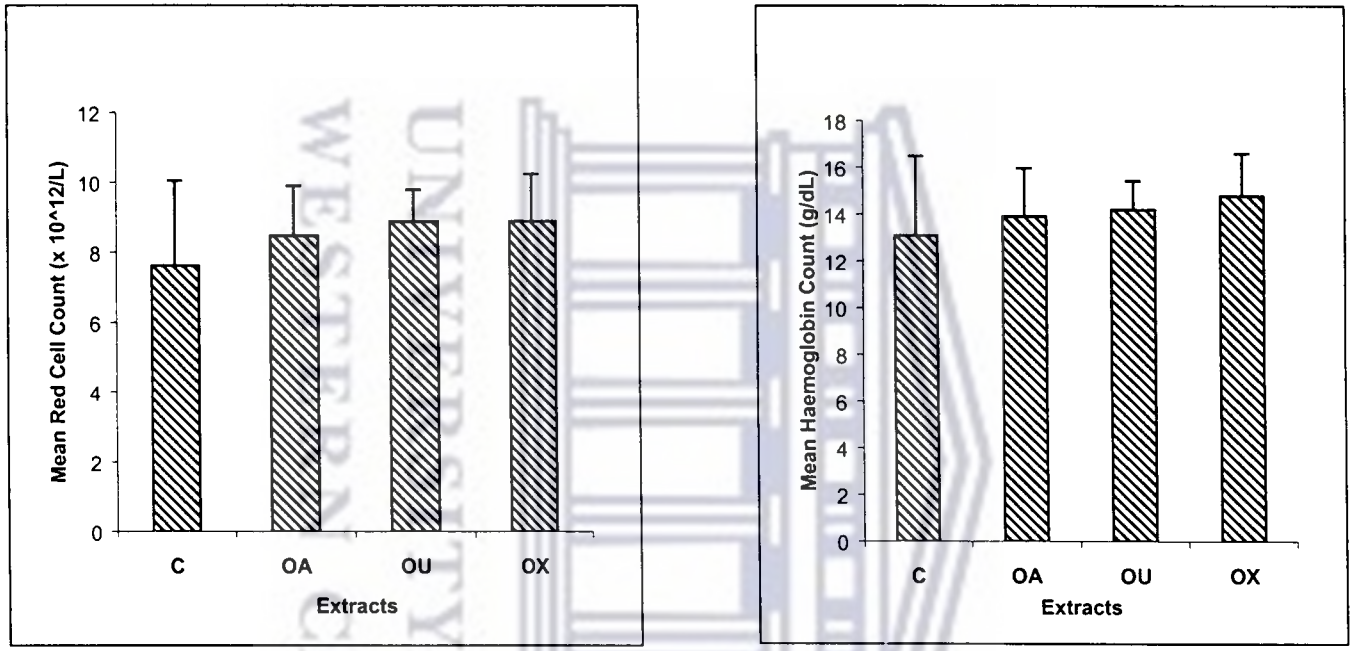


Figure 1. Analysis of (a) Red Cell count and (b) Haemoglobin count. Results depicted as mean + SE. N=6.

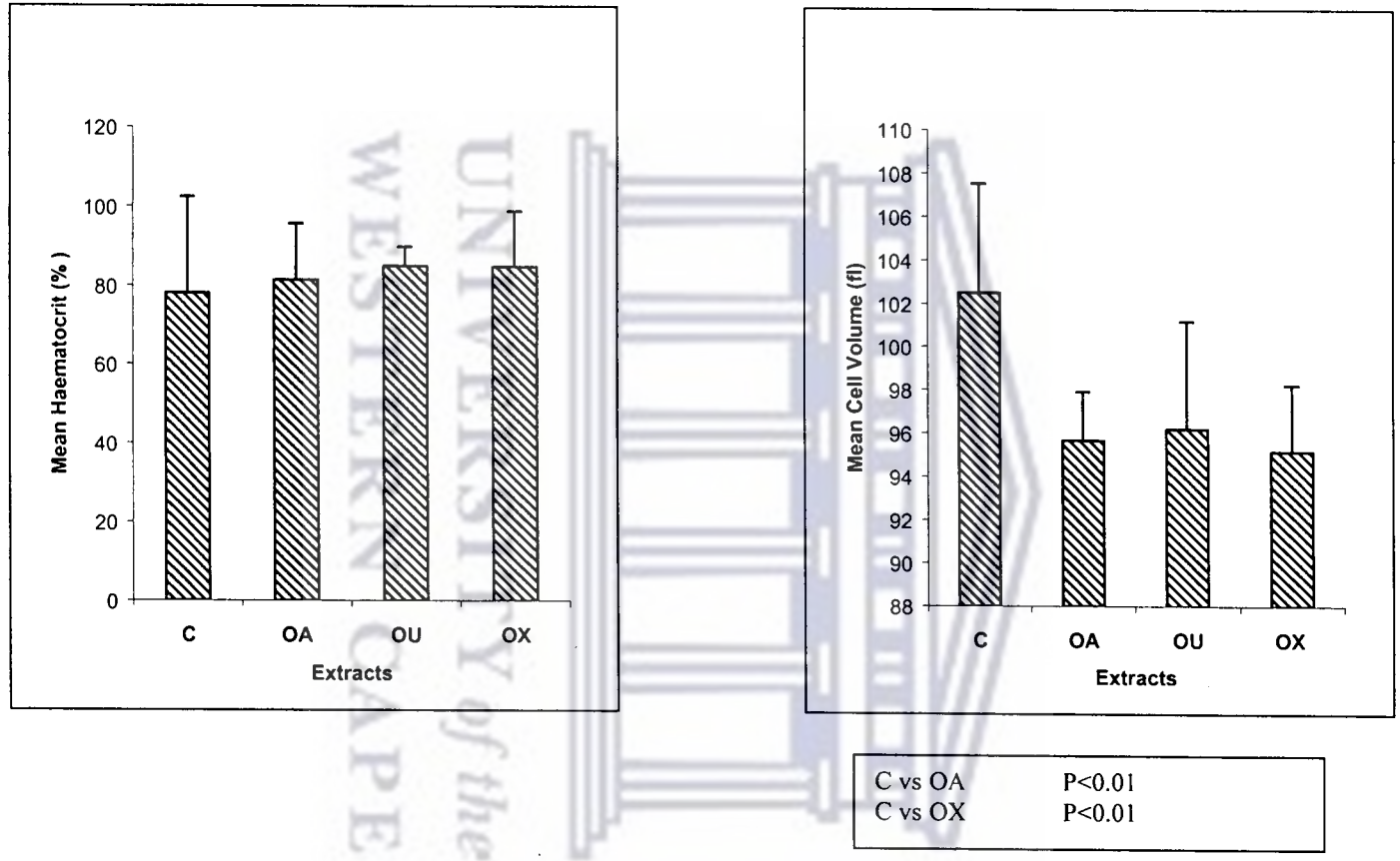


Figure 2. Analysis of (a) Mean Haematocrit levels and (b) Mean cell volume. Included is a key to significant differences in MCV between groups. Results depicted as mean + SE. N=6.

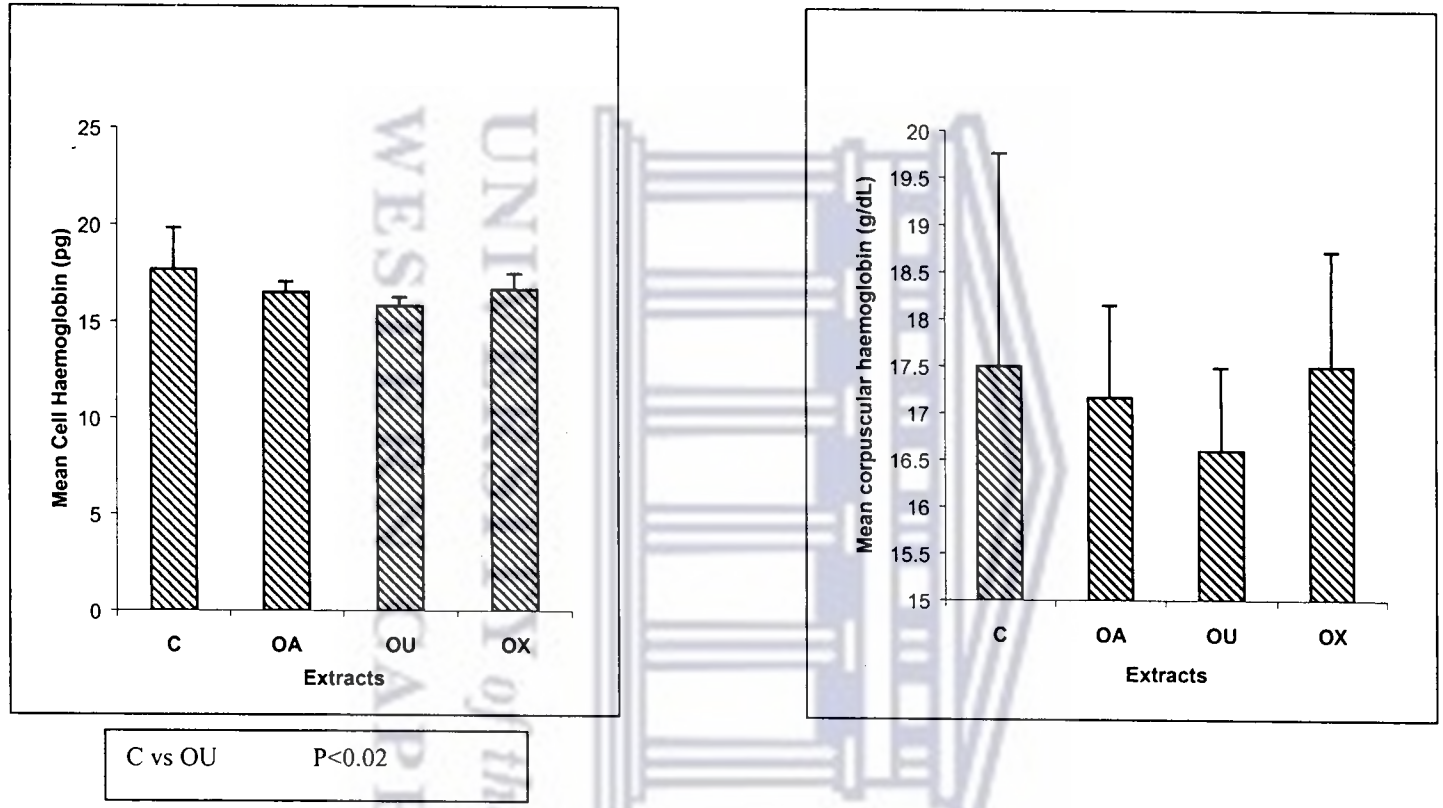


Figure 3. Mean Cell Haemoglobin levels (a) with key of significant differences between groups and (b) Mean corpuscular Haemoglobin. Results depicted as mean + SE. N=6.

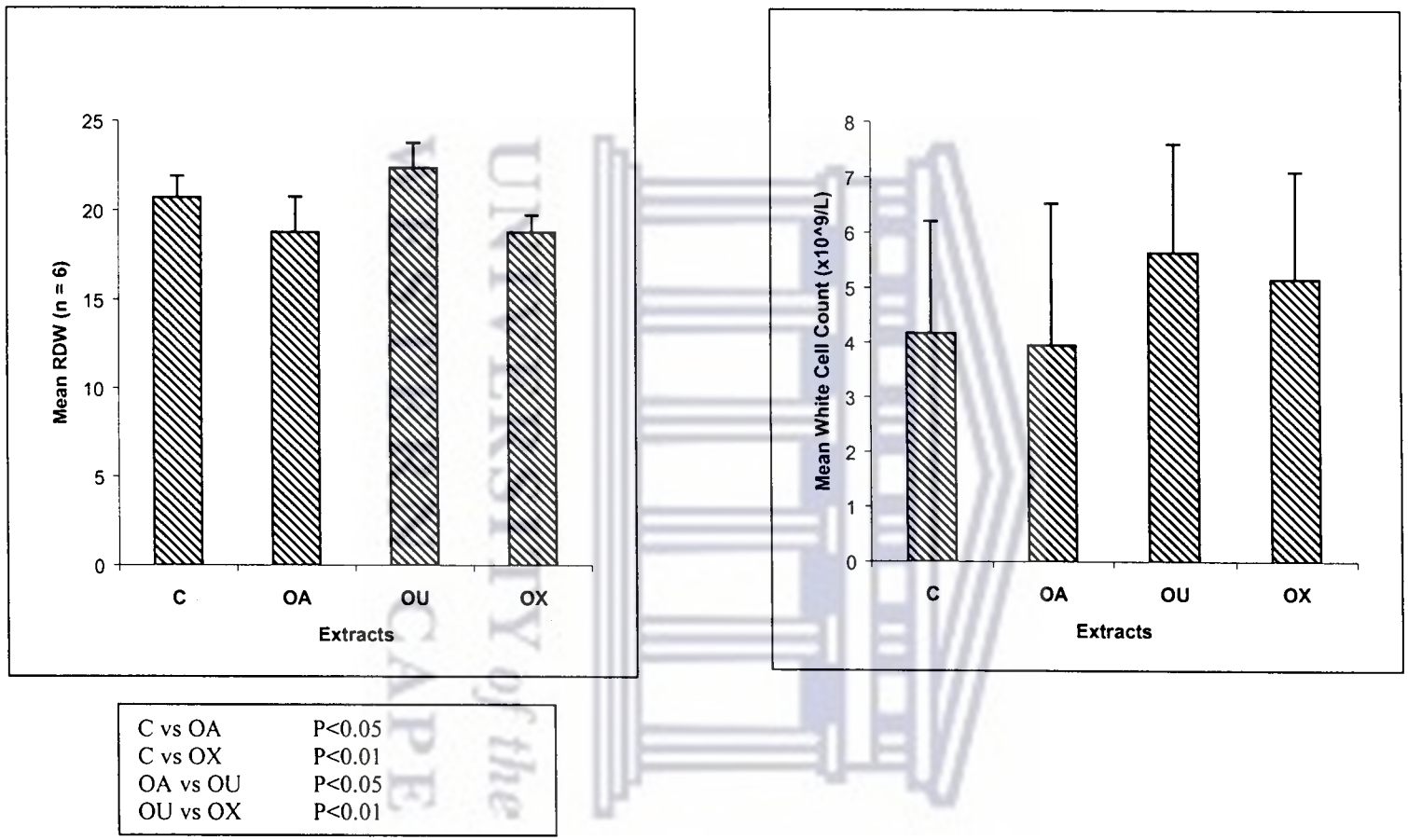


Figure 4. (a) Mean RDW with key of significant differences and (b) Mean white cell count. Results depicted as mean + SE. N=6.

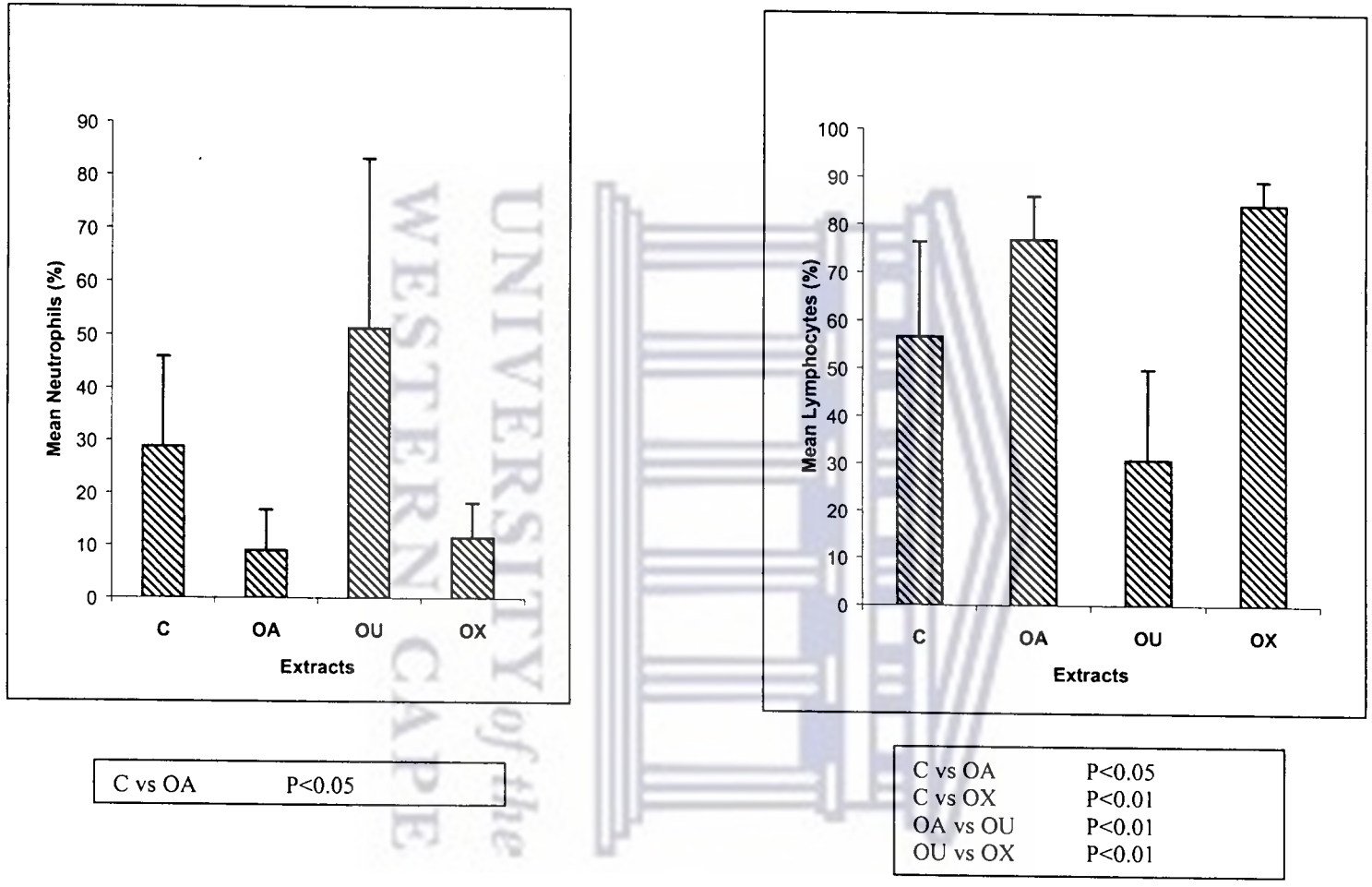


Figure 5. Mean neutrophils (a) and (b) Percentage Lymphocytes. Both with key of significant Wilcoxon plots. Results depicted as mean + SE. N=6.

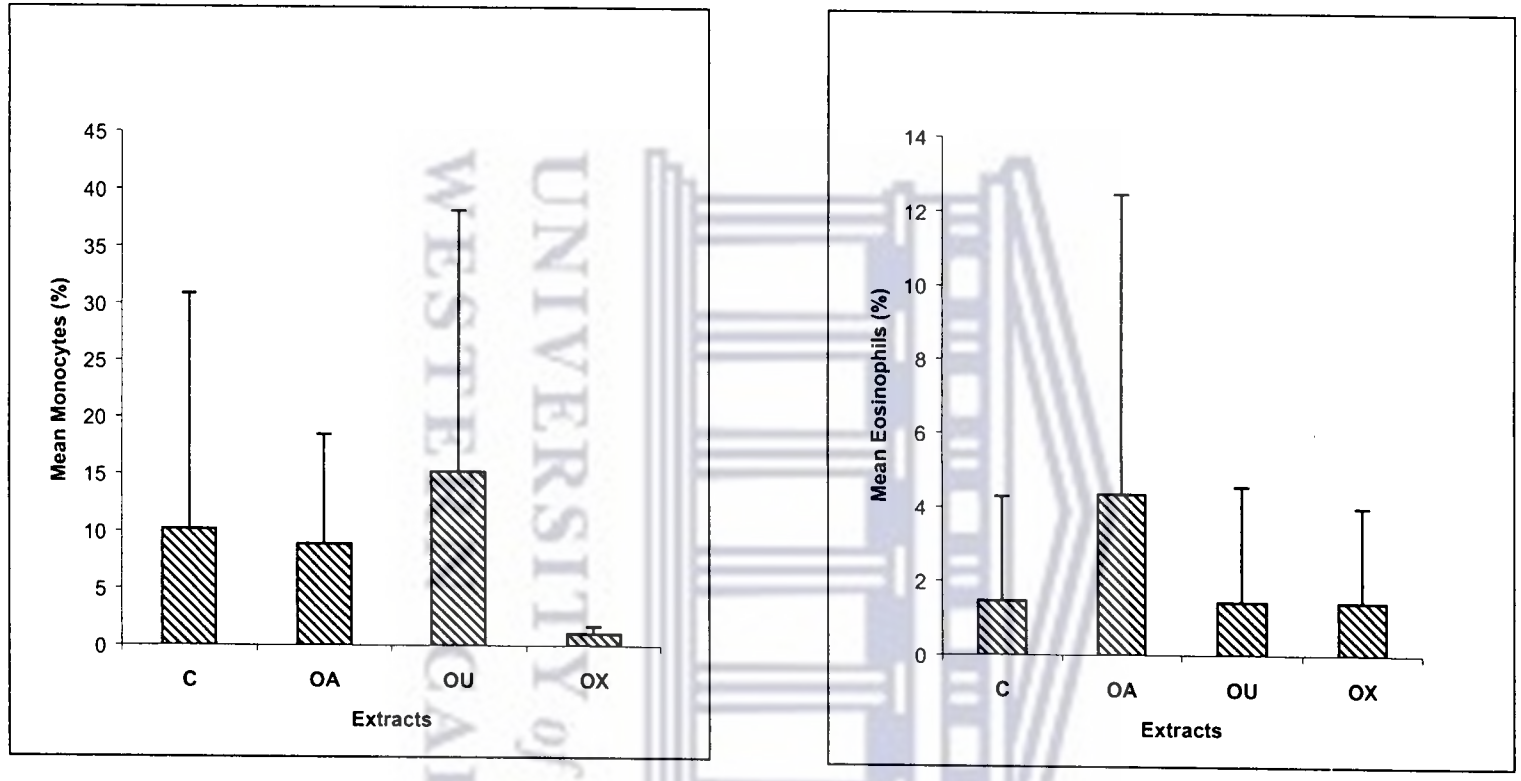


Figure 6. Percentage Monocytes (a) and Eosinophils. Results depicted as mean + SE. N=6.

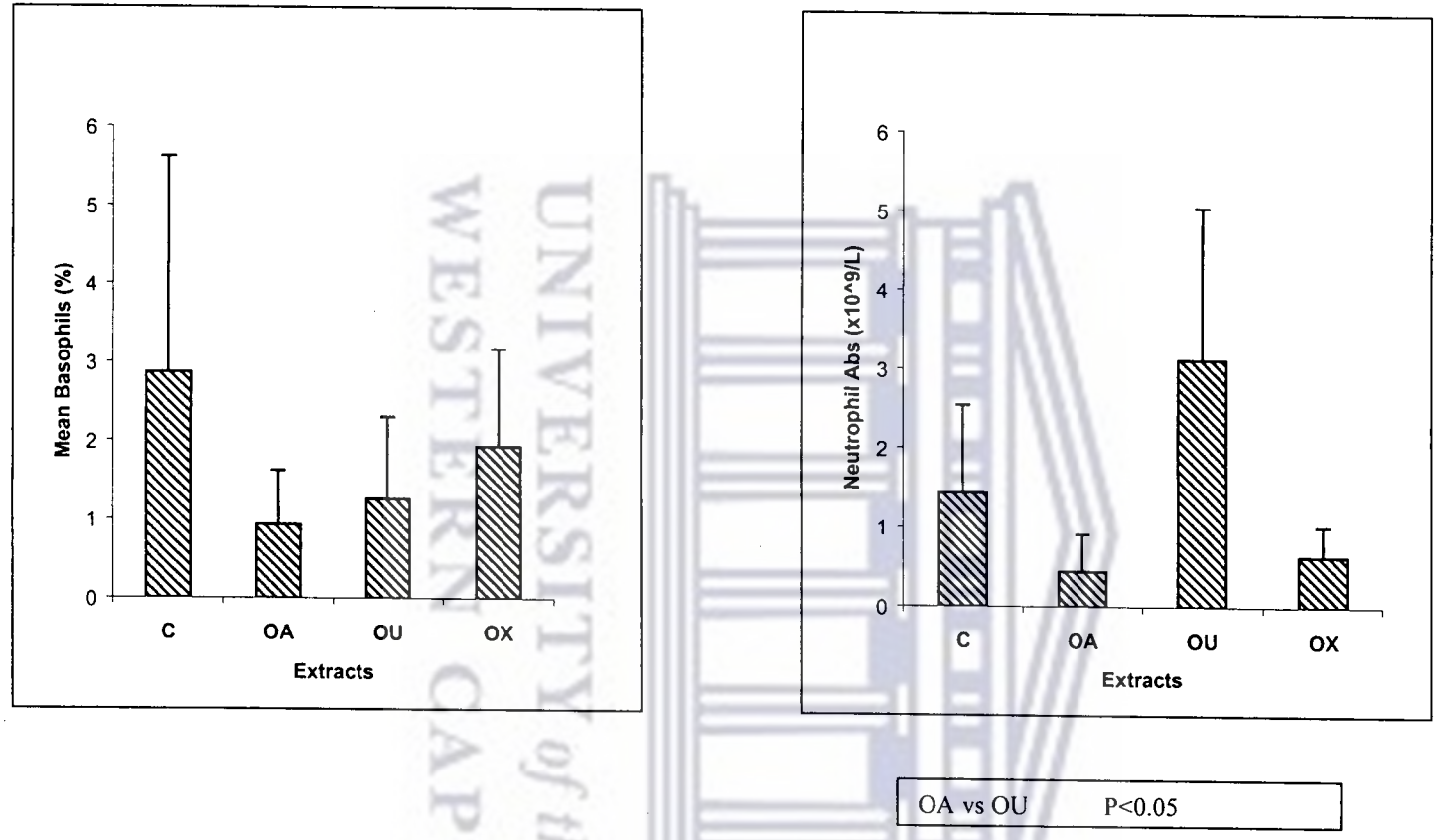


Figure 7. (a) Percentage Basophils and (b) Neutrophil Abs count with key of significant Wilcoxon plot. Results depicted as mean + SE. N=6.

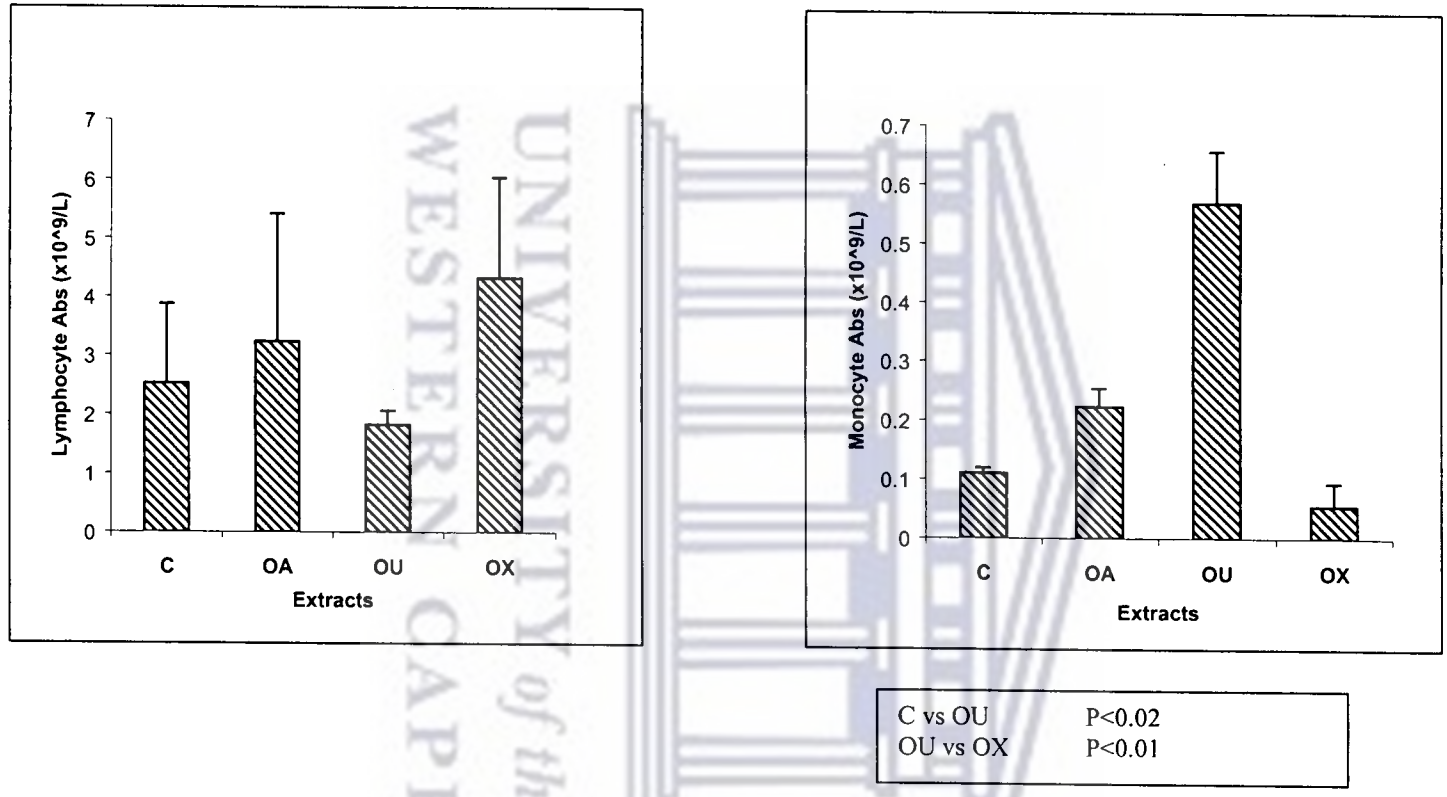


Figure 8. Mean Lymphocyte Abs (a) and (b) Mean Monocyte Abs with Wilcoxon plot of significant differences. Results depicted as mean + SE. N=6.

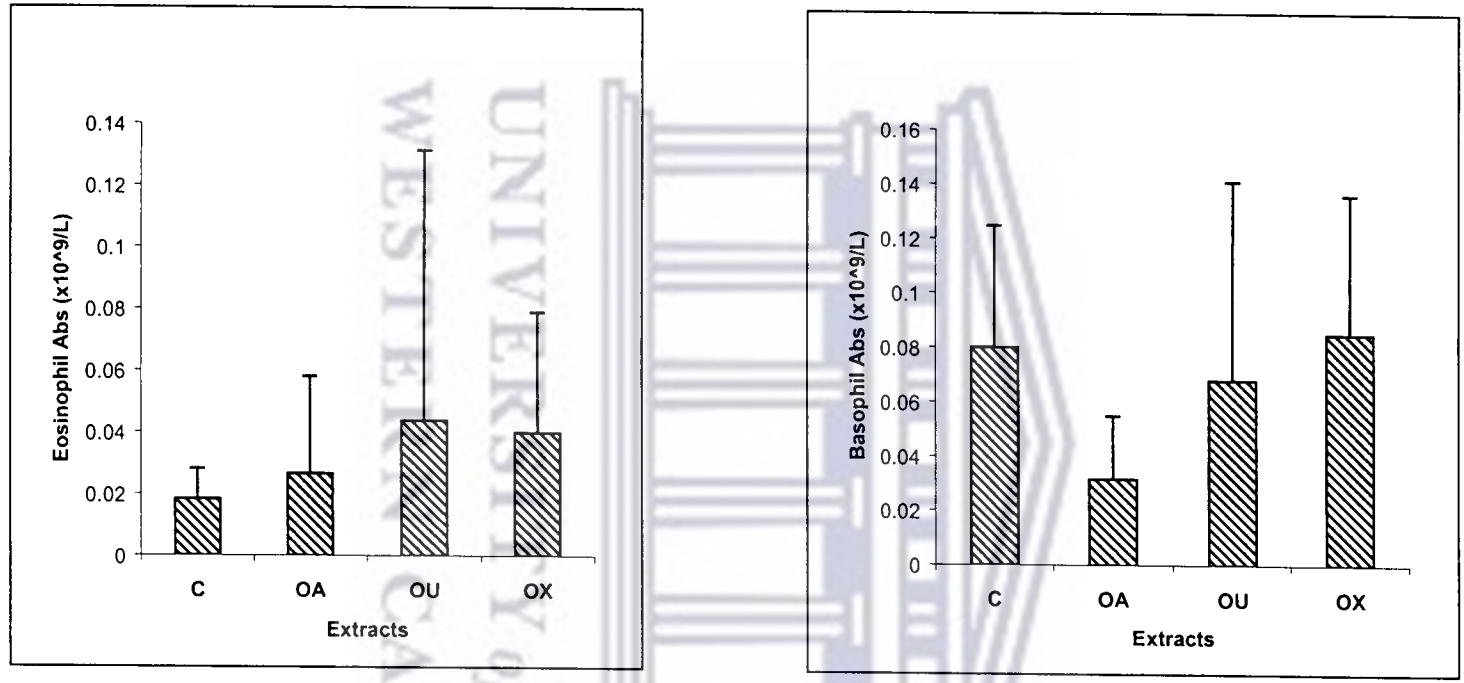


Figure 9. (a) Mean Eosinophil Abs and (b) Mean Basophil Abs. Results depicted as mean + SE. N=6.

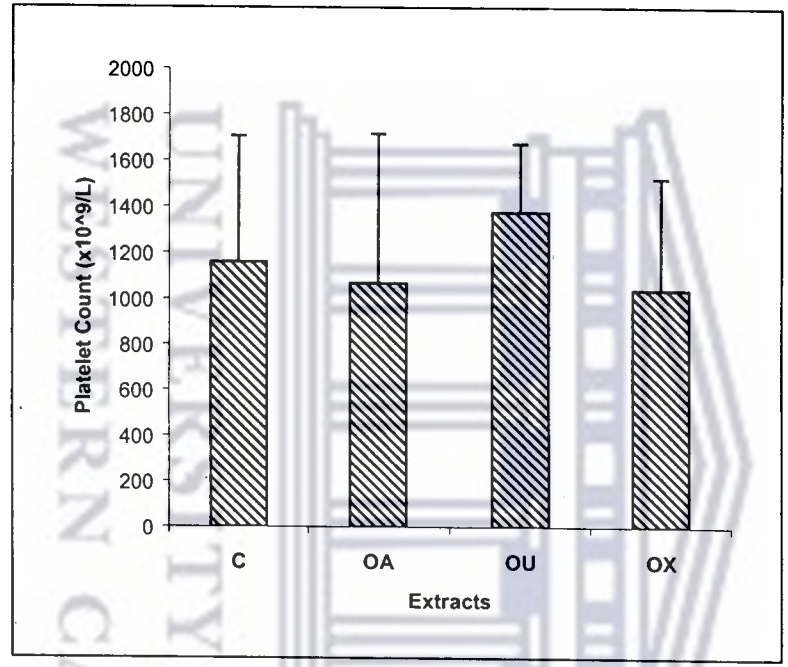


Figure 10. Mean Platelet counts. Results depicted as mean + SE. N=6.

3.4 Tissue histology

Sections obtained from the testes showed significant differences in OA treated animals. These differences are depicted in figure 2.11.

Figure 2.11. Histological sections of (a) control, (b) OA, (c) OU and (d) OX testes. Photos on left taken at x20 and zoomed to x40 on right.

Discussion

In many plant-based contraceptives, inhibition of male fertility after administration of substances has been linked to decrease in sperm density and motility (Kapil and Sharma, 1995). The present study was performed to evaluate the effects of OA, OU and OX on male fertility, and to identify the cause of any visible contraceptive effects. Furthermore, it served as a safety assessment following intraperitoneal administration of these substances for a period of 45 days. An intraperitoneal administration meant that the substances could be passed into the bloodstream of the animals without fear of being metabolized by the digestive system, thus decreasing the amount of time required for observation.

These experiments were also performed to evaluate whether crude extracts of plant leaves containing the active contraceptive substance in known quantities had the same effect as the pure extract found in these leaves or whether their action was possibly masked by other compounds in the ethanol extract. From the studies on fertility and mating behaviour of the extract fed males and pregnancy rates observed in females mated with them, it is evident that the administration of these extracts at the tried doses can induce sterility in the male albino mice.

The results reflect an inhibitory effect of one of the crude extracts and the pure contraceptive drug on sperm production and development. This is located specifically at the testicular level, where histological sections showed a breakdown in the development of germ cells, particularly at the tight junctional complexes, which have shown to be non-existent in OA treated subjects. Histological sections from OU also reveal a breakdown occurring in treated animals, although not as severe as OA treated animals. Animals treated with OU did not show a significant enough difference to the control with regard to testicular histology, although a contraceptive effect was noted in 66% treated mice. OX did not show any significant changes to that of the control with regard to testes histology, but did reveal a contraceptive effect in 33% of treated mice.

The haematology studies revealed that, although there was a significant difference in neutrophil levels between the control and OA treated animals, they both fell within the normality range (Harkness and Wagner, 1996). However, it was noted that the neutrophil levels of OU, although not significantly different to the control, fell outside the normal range. This suggests that possible bacterial infections or inflammation occurred with the treatment of the OU extract. Also important to note was the sharp decrease in the lymphocyte levels of OU animals. Although there were significant differences in comparison to the control, the other treated groups fell within the normality range. These, coupled with the fact that the neutrophil levels were beyond the normality range suggest that the immune system of the animals were not producing sufficient antibodies to combat infection as a result of either bacterial, mechanical or chemical trauma.

It is also of concern that the mean cell volume, mean cell haemoglobin and RDW changed significantly in the treated groups. These suggest an onset of anemia, and only longer trials with more subjects and greater doses can lead to a conclusion as to the absolute safety and efficacy of the crude extracts.

This study has gives an indication to the safety of utilizing crude plant extracts as contraceptives as is observed by the unaltered body weight. It however, does indicate that certain blood cell parameters may be compromised in the process and that further toxicology studies need to be undertaken to verify the safety of these crude extracts.

It is thus the conclusion of this investigation that the crude extracts from the leaves of *Olea europaea* have an inhibitory effect on the fertility of male mice, much like the isolated chemical contraceptive, oleanolic acid, exerting an antifertility effect in male mice mediated through the testes. The effect of this extract on blood parameters, however, may be a potential immune system challenger, but is inconclusive from this study. It is also appended that more trials be carried out at a level zooming in on the testicular tight junctions to identify possible mechanisms for the observed contraceptive effects. It is also evident that the extract from *Olea exasperata* has no significant effect on the fertility of mice, suggesting that other molecules in the extract may mask the

oleanolic acid within this crude extract. It is also evident that longer studies with more animals should be conducted to establish a more accurate conclusion with regards to the safety and efficacy of using the above plant extracts as male contraceptives in mice.

REFERENCES

1) **Berne, R.M. and Levy, M.N.** (2000). *Principles of Physiology*.3rd edition. Mosby Inc. 590-595.

2) **Fox, S.I.** (1996). *Human Physiology*. 5th edition. WCB Publishers. 112 –140 & 608-628.

3) **Garcia-Granados, A., Martinez, A., Moliz, J.N., Parra, A. and Rivas, F.** (1998). Molecules. [Online]. Available http://www.mdpi.org/molbank/m0087.htm

4) **Granger, S and Scott, I.** (1998). Skin care compositions containing a polycyclic triterpene carboxylic acid and a retenoid. United States patent no. 5,723,139

5) **Harborne, J.H.** (1983). Phytochemical methods. WCB Publishers.

6) **Harkness, J.E. and Wagner, J.** (1996) The biology and medicine of rabbits and rodents. 3rd Ed. Mosby Inc.

7) **Hoffmann, D.L.** (2002). Herbs, Fertility and Contraception. [Online]. Available http://www.stopgettingsick.com/Conditions/condition_template.cfm/1382/69/16

8) **Kapil, A. and Sharma, S.** (1995). Effect of Oleanolic Acid on Complement in Adjuvant- and Carrageenan-induced Inflammation in Rats. *J. Pharm. Pharmacol.* 47: 585-587.

9) **Kong, Y.C., Xie, J. and But, P.P.** (1986). Fertility Regulating Agents from Traditional Chinese Medicines. *Journal of Ethnopharmacology*. 15: 1-44.

10) **Liu, J.** (1995). Pharmacology of oleanolic acid and ursolic acid. *Journal of Ethnopharmacology*. 49: 57-68

11) **Matthews and Van Holde** (1992) Biochemistry. 3rd Edition. Mosby Inc. 680-726.

12) **Muto, Y., Ninomiya, M. and Fujiki, H.**(1990). Present status research in cancer chemoprevention in Japan. *Japanese Journal of Clinical Oncology*. 20: 219-224.

13) **Phillipson, J. D.** (2001). Phytochemistry and medicinal plants. *Phytochemistry*. 56: 237-243.

14) **Rajasekaran, M., Bapna, J.S., Lakshmanan, S., Ramachandran Nair, A.G., Veliath, A.J. and Panchanadam, M.** (1988). Antifertility effect in male rats of oleanolic acid, a triterpene from Eugenia jambolana flowers. *Journal of Ethnopharmacology*. 24: 115-121.

15) **Rhoades, R and Pflanzer, R.** (1996). *Human Physiology*. 3rd edition. Saunders College Publishing. 712-720.

16) **Sherwood, L.** (2001). Human Physiology: From cells to systems. 4th edition. WCB. 368-386.

17) **Tortora, G.J. and Anagnostakos, N.P.** (1987). The reproductive systems. *Principles of Anatomy and Phyisiology*. 5th edition: 705-713

18) **Tokuda, H., Ohigashi, H., Koshimizu, K. and Ito, Y.** (1986). Inhibitory effects of ursolic acid and oleanolic acid on skin tumor promotion by 12-O-tetradecanoylphorbol-13 –acetate. Cancer letters.33, 279-285.

19) **Xu, S.L.**(1985). Effects of oleanolic acid on chronic hepatitis: 188 case reports. *Symposium on oleanolic acid*. 23-25.

ELECTROPHYSIOLOGY

CHAPTER 3

MATERIALS AND METHOD

3.1 General Introduction

In order to understand the mechanisms of ionic transport in epithelia, it is important to determine the intracellular potentials of the cells with reference to the respective membranes, namely the apical and basolateral membranes. The transport of ions generates a transepithelial potential difference or voltage (Vt) and the transported ions influence the transport of fluids across epithelia.

This study makes use of the electrophysiological technique of intracellular recordings via the microelectrode puncture technique. This method is used to determine the resting membrane potential (RMP) of the apical membrane (Va) and the RMP of the basolateral membrane (Vbl). The transepithelial resistance of cultured TM4 Sertoli cell monolayers in reference to the bath medium was, however, measured using an EVOM transepithelial voltohmeter (World Precision Instruments, Inc).

Utilizing the method of intracellular puncturing allowed for the determination of the mechanism of known channel blocking drugs. This gave an indication as to the existence of certain channels and pumps on the respective membranes. It also serves as a reference to the action of medicinal plants extracts on the ionic transport of the epithelia. The transepithelial studies allowed us to measure the integrity of the BTB and the effect of extracts on the development of tight junctions between adjacent Sertoli cells.

3.2.1 Culturing of TM4 Sertoli cell monolayers

The Sertoli cell line was cultured as endorsed by Janecki *et al.* (1991). Janecki *et al.* (1991) showed in previous studies that in primary cultures at least 10^6 cells per $0.64cm^2$ well was required to be cultured over a period of 7 days under the influence of

testosterone (10^{-8}M) and FSH (100ng/ml) under a temperature of 36.5°C in 95% humidity and 5% CO_2. This study made use of this protocol using the TM4 Sertoli cell line.

The TM4 Sertoli cell line was acquired from American Type Cell Culture (ATCC, Rockville, USA). The culture characteristics of this strain are consistent with those of primary cell cultures of Sertoli cells (Mather, 1980). TM4 responds to FSH with an increase in growth and intracellular increase of cAMP but is non-responsive to LH (Tindall *et al.*, 1975). The cells of these cultures contain androgen, estrogen and progesterone receptors and secrete transferrin, H-Y antigen and a novel retinol-binding protein (Mather, 1980).

Upon receipt, the frozen cell line was thawed by rapid agitation in a 37°C water bath for approximately 60 seconds. As soon as the ice was melted, the ampule was removed from the bath and made sterile by being immersed in 70% ethanol at room temperature. The cell suspension was diluted into a culture medium consisting of Dulbecco's Modified Eagles Medium (DMEM) supplemented with 2.5mM L-glutamine, 5% horse serum and 2.5% fetal bovine serum and placed in an incubator with an atmosphere of 95% humidity and 5% CO_2 at 37°C. To avoid excessive alkalinity during the recovery period of the cells, the culture medium was placed into culture flasks and placed in an incubator to allow pH adjustment prior to the addition of the ampule contents. Culture media was renewed every 48 hours.

3.2.2 Subculture of Sertoli cells

For the procedure of subculture, culture media was removed and 2-3ml trypsin EDTA (2.5%) was added to the culture flask. After a period of 2 minutes at room temperature, fresh media was introduced, aspirated and dispensed onto matrigel-coated millipore filters in 2ml quantities. These filters were placed in wells, acting as a bicameral system for electrophysiological experiments (figure 3.1). The rest of the culture was then freeze-dried to ensure that stocks of Sertoli cells were kept.

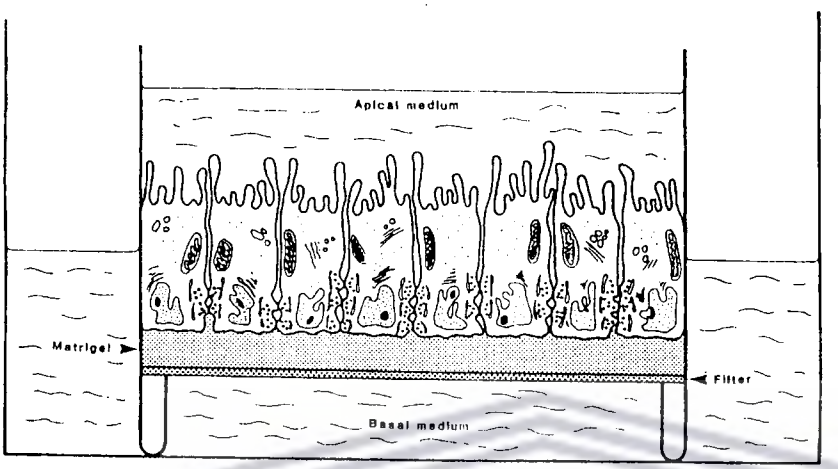


Figure 3.1. Illustration of cells grown in a bicameral system. Taken from Onoda et al. (1990)

3.2.3 The Monitoring of Cell Growth

Cell culture medium containing 10^5 and 10^6 cells was seeded onto matrigel-coated Millipore filters. Cell culture medium (*Appendix A*) for all plates was replaced after an initial 24 hour period and thereafter on a 48-hour basis. The cells were cultured for 7 days during which time they were monitored visually on a daily basis to establish growth patterns.

3.2.4 Histology of cultured cells

After each day, a matrigel filter was removed, immersed in Bouin's solution for 20 minutes and then immersed in ascending grades of ethanol. After being immersed in 80%, 90% and 100% ethanol, the filter was then immersed in xylene and thereafter embedded in hot wax for 24 hours.

Once the 24 hours had passed, the filters were mounted in wax blocks and allowed to solidify. The wax blocks were then sectioned using an "820" Spencer microtome (American Optical Corporation) into 4 μm in thickness and mounted onto slides. The

slides were incubated at 60°C for 24 hours, and then dewaxed in xylene, and hydrated in descending grades of 100%, 90%, 80%, 70% ethanol and water for 5 minutes each.

Once the tissues had been hydrated, the slides were stained in haematoxillin for 15 minutes. The haematoxillin was then removed by briefly rinsing with Scott's tap water. The counterstain, eosin, was then added for 30 seconds, followed by the rinsing with distilled water.

Once this had been done, the slides were then dehydrated once again in ascending grades of alcohol and xylene, allowed to incubate at 60°C for 3 hours and then be covered with Canada balsam and coverslips. The slides were then photographed using a Winder M camera (model: 476-79-9901) for later histological analysis.

3.3.1 Electrophysiological Apparatus and Methodology

The following section explains the apparatus used in the recording of intracellular potentials.

3.3.1.1 The microelectrode

Micropipettes were pulled from borosilicate glass capillaries (GC100F-10, Clark Electromedical Instruments, England) using a microelectrode glass puller (Scientific Research Instruments). The micropipettes were filled with the electrolyte 3M KCl, which ensured a high concentration of ions within the tip, giving the tip a low electrical resistance. This filled pipette was then attached to a chlorided silver wire, thus constituting the microelectrode. Mircroelectrodes made in this way had tip resistances of 20-50 MΩ when tested using the Clampex 8.0 software (Axon Instruments).

3.3.1.2 The indifferent electrode

This is also referred to as the bath or ground electrode. This allowed for a potential difference to be measured between the intracellular environment and the bath fluid. This took the form of agar bridges connecting the basal or apical media to a calomel half-cell to serve as a ground reference for intracellular potential. Agar bridges were strategically placed in either the apical or basal medium to allow for the independent measurement of the apical (Va) and basolateral (Vbl) membranes, respectively.

3.3.1.3 The Preamplifier

This apparatus, also referred to as a headstage was used to pass the voltage signal from the microelectrode to the amplifier without electrical interference to the recorded cell. This headstage had an input resistance of 10^{-13} Ω. During this study, a HS-2 headstage (Axon Instruments: x1L) was used.

3.3.1.4 The Micromanipulator

The micromanipulator (Leitz Wetzlar) in conjunction with a 3D hydraulic micromanipulator (Narishige MO 102) allowed for the puncture of cells to take place remotely without the risk of hand associated vibration.

3.3.1.5 The heating stage

This stage served as a holder for the bicameral chamber system and as a temperature regulator, keeping the cells at physiological temperature. A set of coiled tubes running from the perfusate reservoirs was laid on the heat stage. The temperature was controlled by a Narishige biowarmer (model DK) at 35°C, keeping the cells under normal physiological temperature and keeping the entering Ringers at the same temperature as the bath Ringers.

3.3.1.6 The Perfusate Reservoir

Two 10ml reservoirs were held at specific heights above the bath, ensuring a constant flow of solutions in to both the apical and basal chambers. The height of the reservoirs determined the rate of flow of the fluids. Excessive fluid was pumped out using an electrically controlled vacuum pump. This ensured that bath Ringers was constantly replaced, thus preventing any transient changes in the ionic concentration of the bath Ringers.

3.3.1.7 Microelectrode technique

Cells grown in bicameral chambers were punctured with a microelectrode to measure the voltage between the bath and across a single TM4 Sertoli cell. For the measurement of the apical RMP the following setup was used. A bath electrode in the form of an agar bridge was attached to a calomel half-cell, and immersed in the basal compartment of the bicameral system. This served as the apical medium reference. A recording microelectrode impaled across a cell measured Va. Drugs were administered via the bath medium and the effect of these drugs on RMP recorded. For the study of the basolateral membrane potential, the bath electrode was attached to the basal medium.

3.3.1.8 Output Devices

A dual trace oscilloscope (model LBO-514; Leader) was used to display electrical traces. This oscilloscope was, however, only used as a second reference as the circuitry was connected to a PC running Clampex 8.0 (Axon Instruments, Inc) software. All data was monitored via the PC, which allowed for it to be stored digitally and accessed at any time.

An Axoclamp-2A system (Axon Instruments. Inc.) allowed for the recording of intracellular potentials, which was constantly monitored via a digital panel meter. The voltage output was also connected to an audio monitor, which responded to increases in voltage by an increase in frequency. This allowed the apparatus to have an audible indication to the moment of contact between the cell membrane and the tip of the microelectrode.

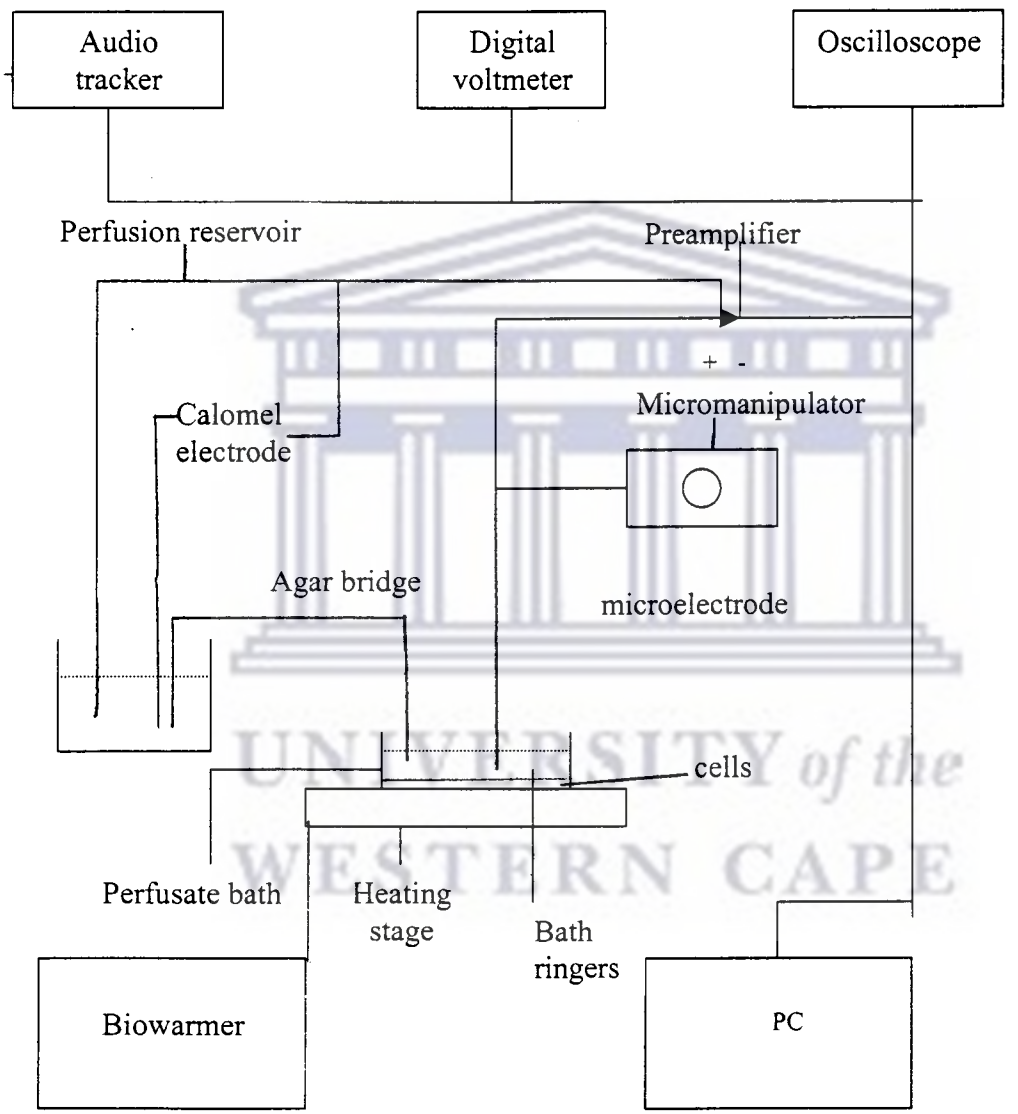


Figure 3.2. Schematic representation of the apparatus used in micropuncture studies

3.3.2 Vibration

Placing the electrophysiological set-up on a solid concrete surface minimized ground or surface vibration. This concrete surface in turn, was covered by a 2.5cm thick rubber, which further dampened any floor-derived vibration.

3.3.3 Noise

Low electrical noise is essential to recording small changes in voltage. Aside from the electronics, the dominant sources of noise were AC picked up by the electrode holder and the microelectrode. Housing the apparatus in an earthed Faraday cage reduced this electrical noise. Surge free AC sockets were supplied to all laboratory equipment to further reduce noise. Earth loop noise was avoided by linking all earths to one point on the amplifier.

PC monitors can create a lot of noise and are difficult to shield. Since the monitor used for this study was relatively old, it was located a distance away from the setup so as to minimize noise.

The input connectors on the headstages are a critical component in the recording circuit. Dirty or corroded connectors are possible sources of noise. To prevent this, gold-plated brass connectors were used and kept clean to avoid salt crystals from forming on the connector.

3.4 Electrophysiological Experiments

3.4.1 Protocol for measuring the effects of different concentrations of extracts on the transepithelial resistance of cultured TM4 Sertoli cells

The cells were allowed to grow for 7 days. There were 24 wells per plate, each containing a 0.45 μm Millipore filter. In each of these chambers, 1ml medium (Janecki *et al.*, 1991)

containing 10^6 cells were seeded onto the filter. As comparative study 10^5 cells were seeded onto 10 filters. This bicameral system simulates the environment of the seminiferous tubules (Onoda *et al.*, 1990) by allowing for both the apical and basolateral membranes of the cells to be exposed to Ringers. The cells were allowed to incubate overnight, after which the growth medium was replaced. Thereafter, the medium was replaced every 48 hours. The cells were cultured for 7 days, Rt determined on each day.

3.4.2.1 Protocol for measuring transepithelial resistance

The transepithelial resistance was measured daily over a 7-day growth window using an EVOM transepithelial voltmeter (World Precision Instruments). The Rt of empty Millipore filters was subtracted from Rt measured over chambers containing known quantities of cells and standardized to cm^2.

3.4.2.2 Protocol for establishing the control: Pure Ringers vs 0.1% ethanol

At day 8, the chambers were divided into the different experimental groups. It was established by De Kock (1989) that ethanol can be used in concentrations below 0.3% without having any toxic side effects on cultured cells. Since the crude extracts in this project are totally insoluble in water (Harborne, 1983) it was necessary to firstly dissolve OA 0.1% ethanol Ringers.

3.4.2.3 Protocol for testing the effects of crude extracts on Rt and V

Oleanolic acid (OA) has been shown to induce apoptosis in cultured Sertoli cells when administered in doses of 50µg/ml and found to be ineffective at high concentrations (Bennet *et al*, *2000*). Since this compound is highly insoluble in water it was made up in Ringers with 0.1% ethanol. Final concentrations of ethanol in both the Ringers containing purified compound or crude extract amounted to not more than 0.1%.

Pure OA was added to the chambers in concentrations of 50 µg/ml, 100 µg/ml and 200 µg/ml. These experiments were done in duplicate. Simultaneously, concentrations of OU

and OX crude extracts containing the above amounts of OA were administered to chambers. The constitutions of the crude extracts are detailed in *Appendix B*. After 24 hours incubation, the transepithelial resistances and potentials were measured. If any change was noted in the transepithelial resistance, the dosage at which this effect was noted was used for intracellular recordings.

3.4.2.4 Protocol for measuring intracellular recordings

Glass electrodes were filled with 3M KCl had tip resistances ranging between 20 to 50 MΩ. Signals were amplified and recorded using Clampex 8.0 software. Intracellular recording times varied from 5 minutes to 130 minutes. For each series of experiments, cells from at least 6 culture chambers were studied.

For basolateral membrane studies, the indifferent electrode was housed in the basal chamber, while apical membrane studies required the indifferent electrode to be attached to the apical medium. Upon impalement of cells, change in resting membrane potential was signaled via an audio monitor. Once the recording potential stabilized, solutions containing different blockers, crude extracts and oleanolic acid were introduced to the perfusate reservoir and allowed to flow into the bathing chamber.

3.4.2.5 Protocol for determining the Vi of TM4 cells

The commencement of intracellular recordings on the bicameral system started 24 hours after the administration of the tested substances. A chamber was removed from the plate and immersed in a petri dish containing Ringers made up as an ionic equivalent of DMEM (Table 3.1). The cultured monolayer of Sertoli cells then underwent membrane puncture as per protocol at 36.5°C (Janecki *et al.*, 1991).

The extract concentrations used were the result of the predetermined dosage study i.e. concentrations at which transepithelial resistances were affected. If a response was noted by a difference in the intracellular potentials of controls and experimental groups, the

Ringers medium was replaced with chloride free Ringers, and subsequently, Na^+ free and K^+ free Ringers, depending on the resultant response to the new media. The medium was changed to establish whether the response is in relation to chloride, sodium or potassium ion movement across the membrane.

3.5 Composition of solutions

The solutions were designed to approximate the fluid in the seminiferous tubule. The compositions of the solutions are tabulated in figure 3.1.

Distilled water was used to make up the solution to 1 litre. Ringers were adjusted with mannose to an average osmolality of 320 osm/L for the perfusate using a calibrated vapour pressure osmometer (model 5100c, Wescor Inc.). Solutions were adjusted with NaOH or HCl to maintain the pH at 7,4 prior to perfusion. Table 3.1 gives a summary of the compositions of the perfusion solution used.

Once a stock solution of Ringers was made up, experimental drugs were added in the following concentrations. Furosemide (10^{-6}M) (Baldrick *et al.*, 1988), Bumetanide (10^{-6}M) (Ko *et al.*, 1998), Acetazolamide (10^{-6}M) (Cuthbert and Wong, 1975), $BaCl_2$ (2mM) (Weltens *et al.*, 1992) and Amiloride (10^{-6}M) were added and dissolved in the Ringers.

Table 3.1 ***Standard Ringer Solutions for intracellular studies***

Salt	Normal (mM)	Cl^- free (mM)	K^+ free (mM)	Na^+ free (mM)
NaCl	123	-	123	-
KCl	5	-	-	5
$MgCl_2$	1	-	1	1
$CaCl_2$	1	-	1	1
$NaHCO_3$	25	25	25	-
NaH_2PO_4	1.2	1.2	1.2	-
Na acetate	1	1	1	-
$NaSO_4$	-	123	-	-
K_2SO_4	-	5	-	-
$CaPO_4$	-	1	-	-
Choline chloride	-	-	-	123
H_2CO_3	-	-	-	25
Glucose	5.5	5.5	5.5	5.5
Total: Na^+	150.2	150.2	150.2	-
Total: K^+	5	5	-	5
Total: Cl^-	130	-	130	130

3.6 Aeration and Temperature Regulation During Perfusion

An air mixture consisting of 95% oxygen (O_2)/5% Carbon dioxide (CO_2) was used to maintain the pH of the bath Ringers at 7.4. This air mixture aerated the bath Ringers above the perfusion bath in a reservoir, averting the damage of the fragile cells by bubbling air into the perfusion bath.

3.7 Drug Administration

Once stable RMP was achieved, the normal bath Ringers was replaced with Ringers containing the experimental drug. Once the effect of the drug on the cells, if any, stabilised on the electrogram, the drug-containing Ringers was replaced with normal Ringers. Following rinsing, recovery was monitored.

3.8 Statistical Analysis

For statistical comparison of the intracellular potentials, a student's *t*-Test was employed. Significance was determined at $P<0.05$. All values are represented as mean ± standard deviation.

For statistical comparison of the transepithelial resistances, non-parametric analysis of variance (Kruskal Wallace) was conducted for all data sets. If any parameters demonstrated significant differences between treatments, then Wilcoxon unmatched pairs analyses were used to establish which treatments were different from one another. Medcalc (version 4.16f – 1997) was used for all statistical analyses.

CHAPTER 4

RESULTS

4.1 Transepithelial Resistances

Cells cultured at 10^5 and 10^6 cells per well developed into monolayers within 48 hours of culture. The transepithelial resistances (Rt) of these cells were maximal between 24 and 48 hours after growth. Figure 4.1 reflects the Rt of the cultured cells over a growth period of 6 days. It was evident that the cells grown in a concentration of 10^5 cells per well developed a greater Rt than cells cultured at 10^6 cells per well over the same culture period. Figure 4.1 best depicts the comparative Rt for cells grown at these concentrations (N=9).

At 24 hours after seeding the cells (day 1) Rt was measured as 385±78.2 $\Omega.cm^2$ for cells grown at 10^5 cells per well and 336.5±84.1 $\Omega.cm^2$ for cells grown at 10^6 cells per well. Rt for cells grown at these concentrations showed no significant differences to each other (P<0.35).

After 48 hours (day 2) Rt was measured as 376±74.1 $\Omega.cm^2$ for cells at 10^5 cells per well, whereas cells grown at 10^6 cells per well had Rt of 344±73.9 $\Omega.cm^2$. Rt for these cells did not differ significantly from each other (P<0.1).

At day 3 the cells measured Rt of 351.9±56.6 $\Omega.cm^2$ for cells grown at 10^5 cells per well and 328.7±49.2 $\Omega.cm^2$ for cells grown at 10^6cells per well. Rt for cells grown at these concentrations showed no significant differences to each other (P<0.08).

At day 4 Rt measured at 318.9±59.3 $\Omega.cm^2$ for cells grown at 10^5 cells per well and 134.3±12.83 $\Omega.cm^2$ for cells grown at 10^6 cells per well. Rt for cells grown at these

concentrations was significantly different from one another ($P<0.05$). Days 5 and 6 showed the same trend.

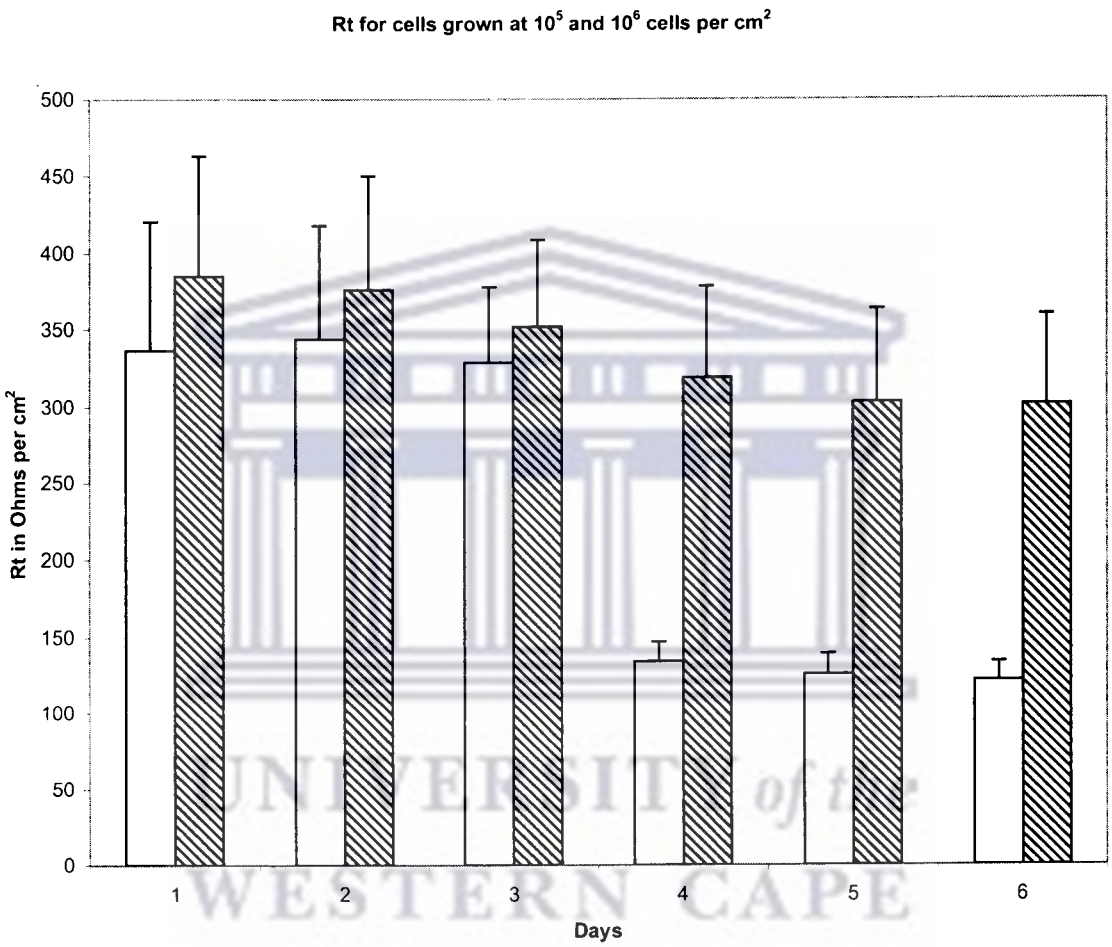


Figure 4.1. Graph of Rt vs Time. Cells grown at 10^6 cells per well represented by clear bars and cells grown at 10^5 cells per well represented by the striped bars.

4.2 Histology of TM4 Sertoli cells grown in the bicameral system

Cells seeded at 10^5 cells per were utilized for histological analysis over a period of 4 days and compared histologically. Two groups of cells were grown in the absence of and with hormone supplementation. Daily cross sections of the developing monolayer on the matrigel surface showed that the cells grown in supplemented media attained confluency within 48 hours. After a further 24 hours, the cells developed into multilayers. Cells supplemented with hormones developed a cuboidal shape. These cells grown in unsupplemented media resembled squamous epithelia in that they were flat and slightly raised at the centre. Figure 4.2 below gives a visual display of the developing cells.

Figure 4.2. Comparative sections over (a) 24 hours, (b) 48 hours and (c) 72 hours for cells in supplemented media (left) and unsupplemented media (right). All photographs taken at 500X magnification.

4.3.1 Intracellular Controls

Upon the impalement of the cell, there was a significant hyperpolarization* in the intracellular potential (Vi) of the cell. In most cases, the impalement profiles had two voltage stages.

The first stage of the voltage profile gave an average reading of –8.5±3.5 mV for the duration of only a few seconds and was unstable. This stage indicated that the microelectrode had touched the surface of the cell membrane. Further micromanipulation resulted in being fully impaled. This was noted by an increase in the magnitude and stability of the recorded Vi. This stable Vi was considered to indicate the RMP. The duration of each experiment ranged from 5 to 130 minutes (N=36). The figure below shows a typical trace, indicating the 2 voltage stages.

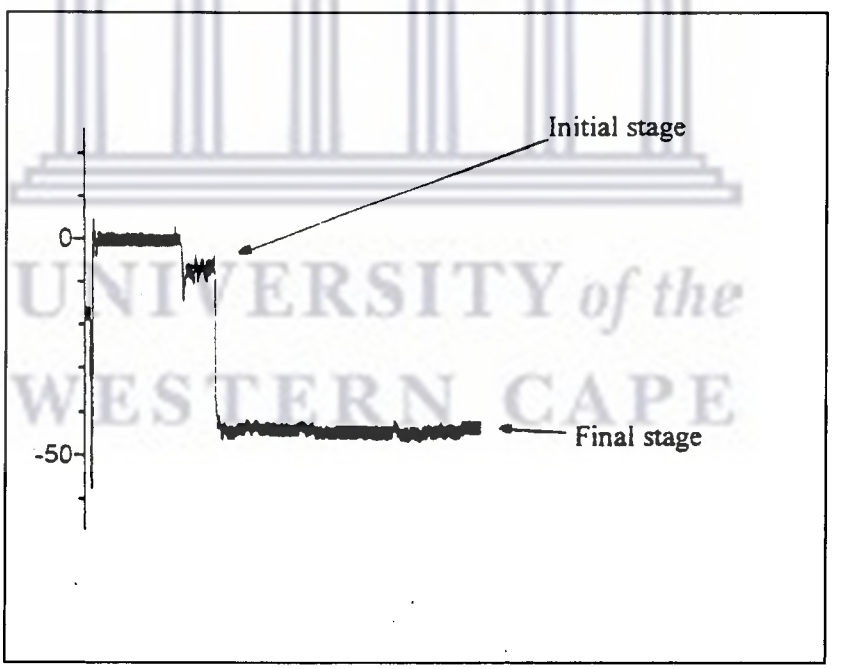


Figure 4.3 Typical trace recorded from a TM4 Sertoli cell.

**hyperpolarization = Intracellular potential (Vi) becomes more negative*
depolarization = Intracellular potential (Vi) becomes more positive

4.3.2 Control Va and Vbl

When a monolayer has attained confluency, it is possible to measure the potential across either the apical or basolateral membrane by simply positioning the indifferent electrode in either the apical medium (Va) or the basal medium (Vbl) (see fig. 3.1).

In this case where confluency has not been attained, the apical media is short-circuited to the basal media via the Millipore filter and therefore Va = Vbl = Vi.

The average control values for the apical (Va) and basolateral (Vbl) potentials were –21.67±1.92 mV (N=36) and –47.46±1.74 mV (N=36), respectively. Paired *t*-Tests showed that Va and Vbl were significantly different from one another ($P<0.0001$). The controls were compared to cells exposed to extracts for a period of 24 hours.

4.4 Effects of Furosemide on the apical and basolateral membranes

Furosemide, a blocker of $Na^+K^+2Cl^-$ co-transport (Fisher, 1997), was administered to the different bath fluids at a concentration of 10^{-6}M to test the hypothesis that chloride co-transporters exist across the apical and basolateral membranes of the TM4 Sertoli cell. The statistically analysed data are graphically presented in figure 4.4 and tabulated in table 4.1, while figure 4.5 illustrates a typical result as digitally recorded by the Clampex program during experimentation.

Under control conditions Va was –21.08±1.28 mV (N=6) in normal Ringers solution. The addition of furosemide caused an immediate depolarization of all the potentials. In the presence of furosemide, Va was depolarized to –6±0.71 mV (P<0.0001). On the replacement of furosemide Ringers with normal Ringers, Va recovered and returned to –20.92±1.43 mV (P<0.83).

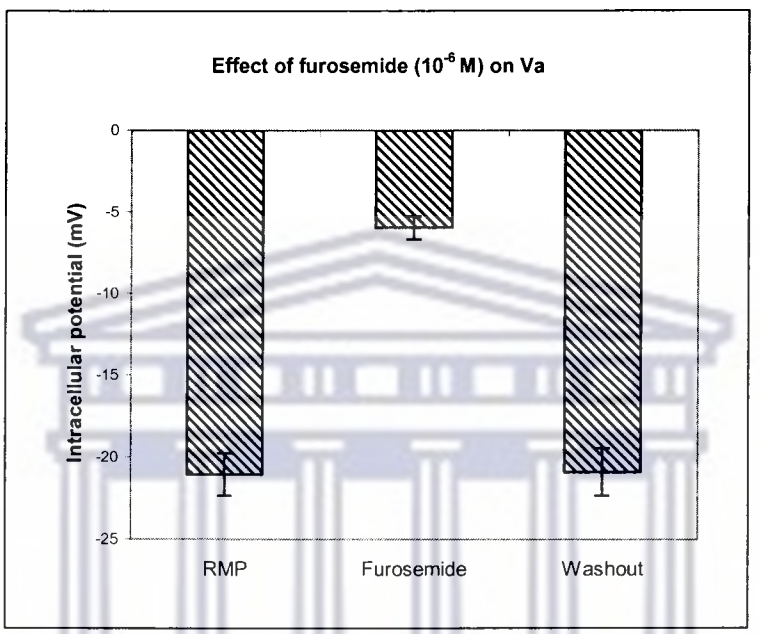
When tested on the basolateral membrane, Vbl depolarized from the RMP of –46.3±0.93 mV to –27.08±0.73 mV (N=6) (P<0.0001). Upon rinsing with normal Ringers, Vbl returned to –46.67±0.88 mV (P<0.54).

Utilising the recorded Va and Vbl data, the theoretical transepithelial resistances (Vt) were calculated using the equation Vt = Va + Vbl. At control values, Vt was –25.25±1.7 mV. Exposure of the cells to furosemide caused a significant depolarization to –21.08±0.98 mV (P<0.001). Upon washout, Vt returned to –25.75±1.87 mV (P<0.67).

Table 4.1 Effect of furosemide on intracellular potential (mV).

	N	RMP	Furosemide	Washout	RMP vs Furosemide	RMP vs washout
Va	6	–21.08±1.28	–6±0.71	–20.9±1.43	P<0.0001	N.S
Vbl	6	–46.3±0.93	–27.08±0.73	–47±2.0	P<0.0001	N.S
Vt	6	–25.25±1.7	–21.08±0.98	–25.75±1.87	P<0.001	N.S

(A)



(B)

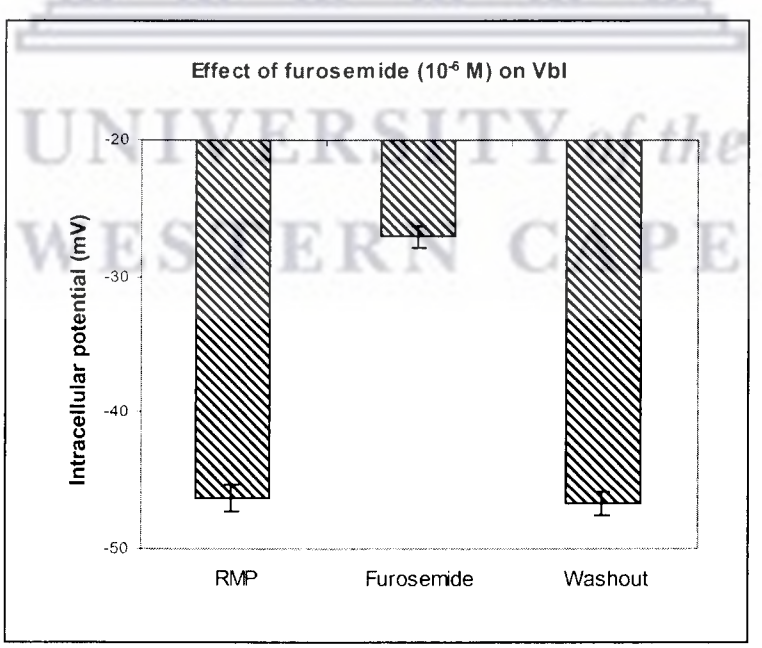


Figure 4.4 Graphs illustrating the effect of furosemide on (A) Va and (B) Vbl. Washout refers to the replacement of experimental Ringers with normal Ringers.

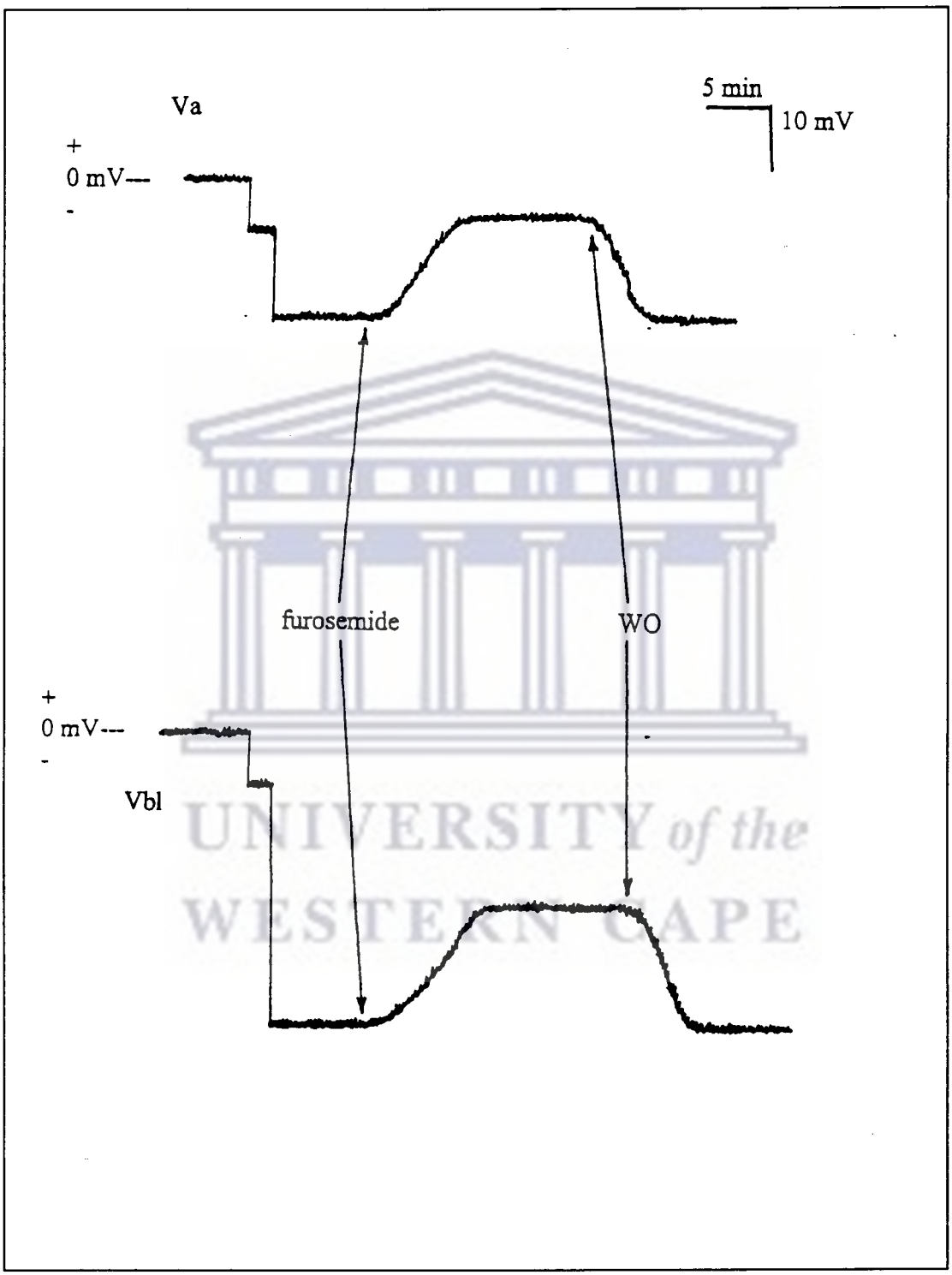


Figure 4.5 The effect of furosemide on Va and Vbl. WO=washout with bath Ringers.

4.5 Effects of Bumetanide on the apical and basolateral membranes

Bumetanide, an inhibitor of the $Na^+K^+2Cl^-$ symport (Palfrey *et al.*, 1983), was administered to both sides of the bicameral chamber at a concentration of 10^{-6}M to test for the presence of $Na^+K^+2Cl^-$ symport activity across the apical and basolateral membranes of the TM4 Sertoli cell. The statistically analysed data are graphically presented in figure 4.6 and tabulated in table 4.2, while figure 4.7 illustrates a typical result as digitally recorded by the Clampex program during experimentation.

Under control conditions Va was –22±2.3 mV (N=6) in normal Ringers solution. The addition of bumetanide caused an immediate depolarisation of all the potentials. In the presence of bumetanide, Va was depolarised to –11.92±1.02 mV (P<0.0001). On the replacement of bumetanide Ringers with normal Ringers, Va recovered and returned to –21.92±2.44 mV (P<0.95).

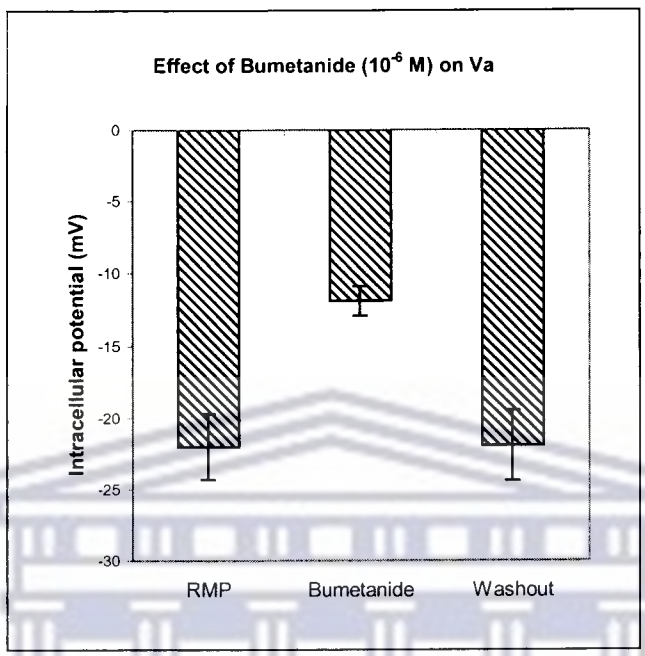
When the basolateral membrane was exposed to bumetanide, Vbl depolarised from the RMP of –48.42±1.88 mV to –39.17±1.37 mV (N=6) (P<0.0001). Upon rinsing with normal Ringers, Vbl returned to –47.92±0.97 mV (P<0.58).

Theoretical Vt was calculated to be –26.42±1.34 mV under control conditions. The administration of bumetanide caused a depolarization to –27.25±0.69 mV that was not significantly different to the control (P<0.25). Upon washout Vt became –26±2.69 mV (P<0.25).

Table 4.2 Effect of bumetanide on intracellular potential (mV). N.S = not significant

	N	RMP	Bumetanide	Washout	RMP vs. bumetanide	RMP vs washout
Va	6	–22±2.3	–11.92±1.02	–21.92±2.44	P<0.0001	N.S
Vbl	6	–48.42±1.88	–39.17±1.37	–47.92±0.97	P<0.0001	N.S
Vt	6	–26.42±1.34	–27.25±0.69	–26±2.69	N.S	N.S

(A)



(B)

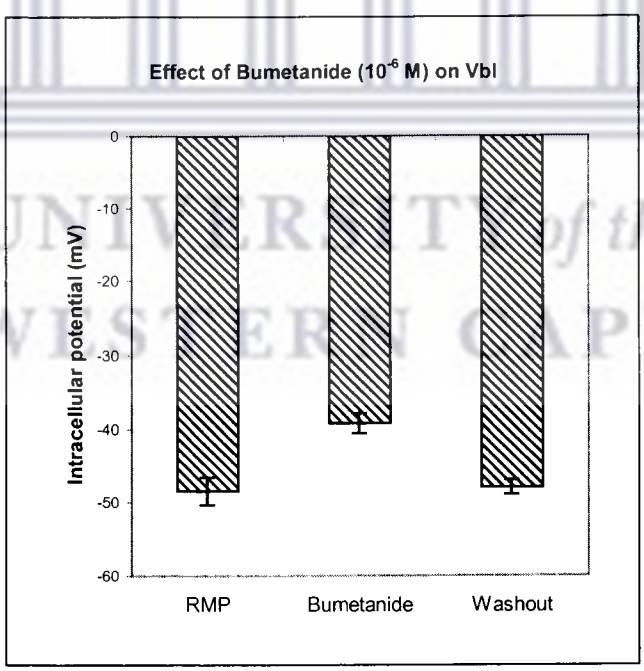


Figure 4.6 Graphs depicting the effects of bumetanide on (A) Va and (B) Vbl.

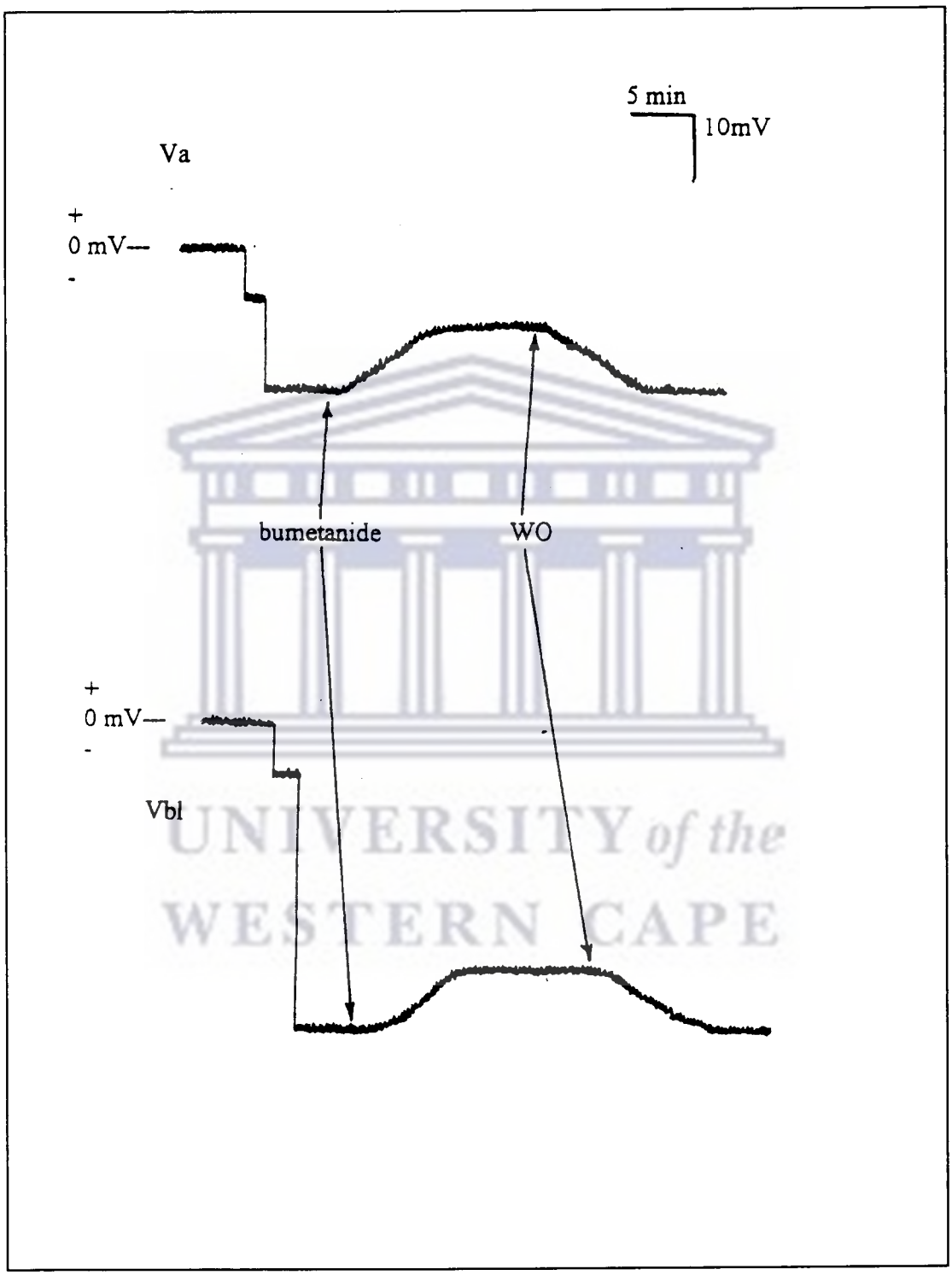


Figure 4.7 The effect of bumetanide on Va and Vbl. WO=washout with bath Ringers.

4.6 Effect of Chloride-free Ringers on intracellular potential

The effects of chloride free Ringers (CFR) on the electrical properties of the TM4 Sertoli cell are summarised in table 4.3 and illustrated in figure 4.8, while figure 4.9 illustrates a typical result as digitally recorded by the Clampex program during experimentation. Under normal Ringers, Va and Vbl were –22.38±0.74 mV (N=4) and –47±0.41 mV (N=4) respectively.

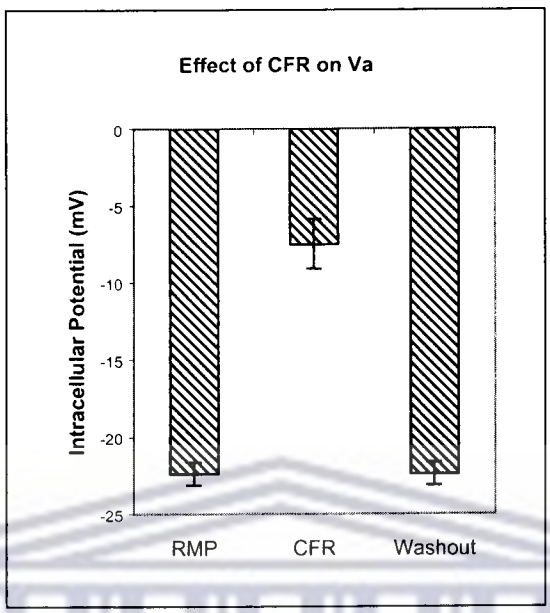
Replacing the normal bath Ringers with CFR immediately hyperpolarised Va to –7.5±1.62 mV (P<0.0001) and Vbl to–35.8±2.25 mV (P<0.002) respectively. Upon washout of the CFR with normal Ringers, both Va and Vbl returned completely to control RMP.

Theoretical Vt was calculated to be –24.63±1.02 mV under control conditions. The administration of CFR caused no significant effect. Cells exhibited Vt of –28.38±3.54 mV (P<0.16). Upon washout Vt returned to –24.63±1.02 mV.

Table 4.3 ***The effect of CFR on potential***

	RMP	**CFR**	**Washout**	**RMP vs CFR**	**RMP vs Washout**
Va	-22.38±0.74	-7.5±1.62	-22.38±0.74	P<0.0001	N.S
Vbl	-47±0.41	-35.8±2.25	-47±0.41	P<0.0001	N.S
Vt	–24.63±1.02	–28.38±3.54	–24.63±1.02	N.S	N.S

(A)



(B)

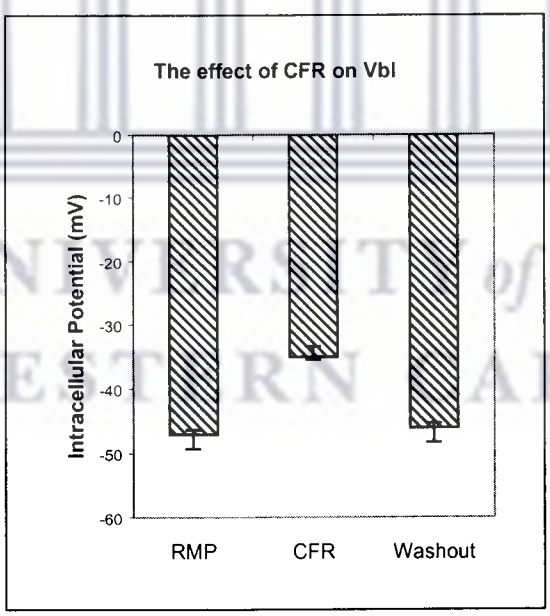


Figure 4.8 The effects of chloride free Ringers on (A) Va and (B) Vbl

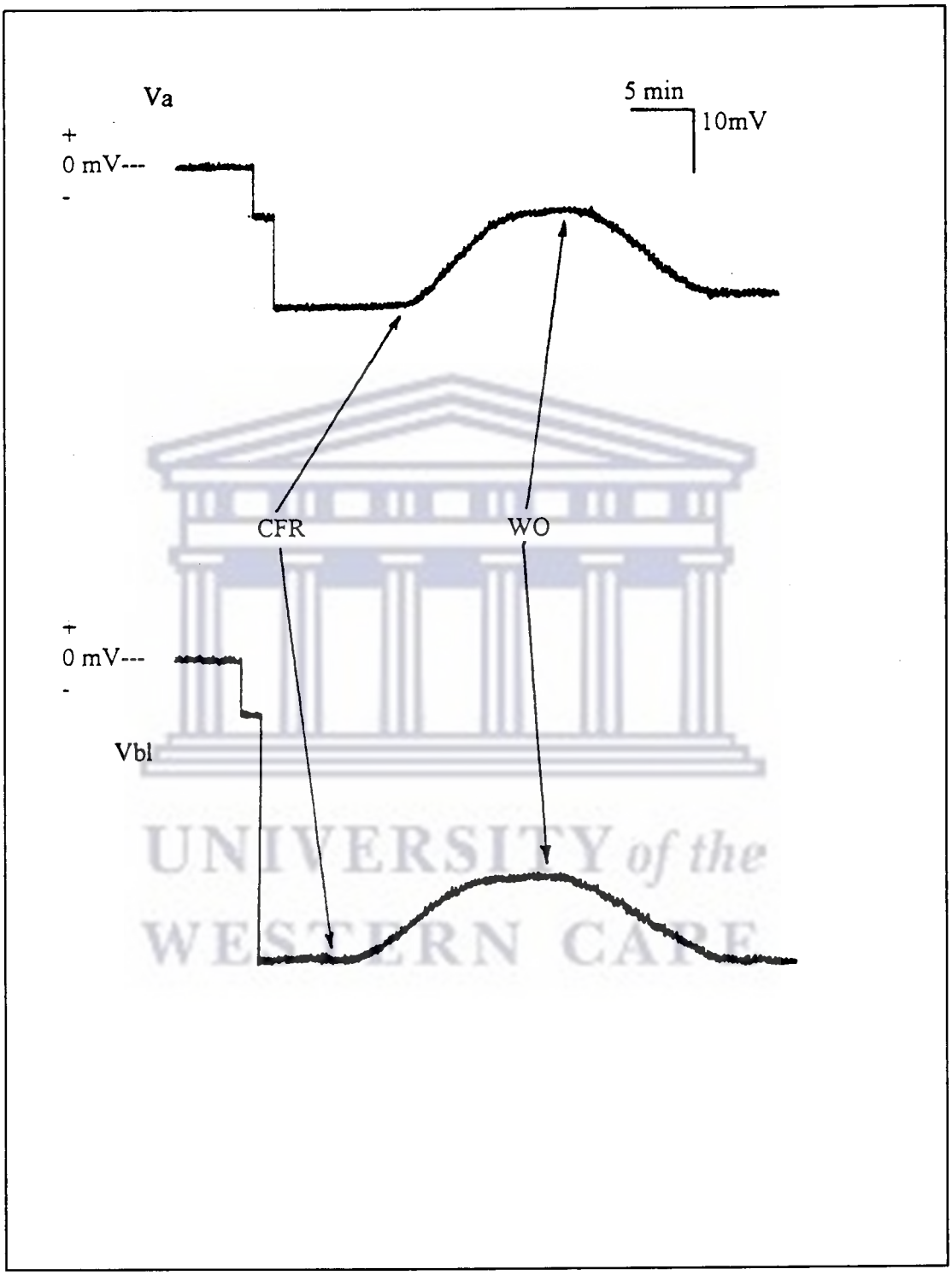


Figure 4.9 The effect of CFR on Va and Vbl. WO=washout with bath Ringers.

4.7 Effect of $BaCl_2$

In an attempt to test for the presence of K^+ channels in the apical and basolateral membranes, $BaCl_2$ was added to the bath while recording the Va and Vbl. The statistically analysed data are tabulated in table 4.4.

Under control conditions Va was –20.5±1.73 mV (N=5) in normal Ringers solution. The addition of $BaCl_2$ caused no change in Va. When tested on the basolateral membrane, Vbl remained unchanged at –45.5±1.89 mV (N=5) throughout the experiment.

Theoretical Vt was calculated to be –25.58±2.09 mV under control conditions. This Vt remained the same throughout the experiment.

Table 4.4 Effects of $BaCl_2$ on Va and Vbl N.S = not significant.

	N	RMP	$BaCl_2$	Statistics
Va	5	–20.5±1.73	–20.5±1.73	N.S
Vbl	5	–45.5±1.89	–45.5±1.89	N.S
Vt	5	–25.58±2.09	–25.58±2.09	N.S

4.8 The effect of K^+ free Ringers

The effect of removing the bath medium's potassium concentration completely is summarised in table 4.5 and illustrated in figure 4.10, while figure 4.11 illustrates a typical result as digitally recorded by the Clampex program during experimentation.

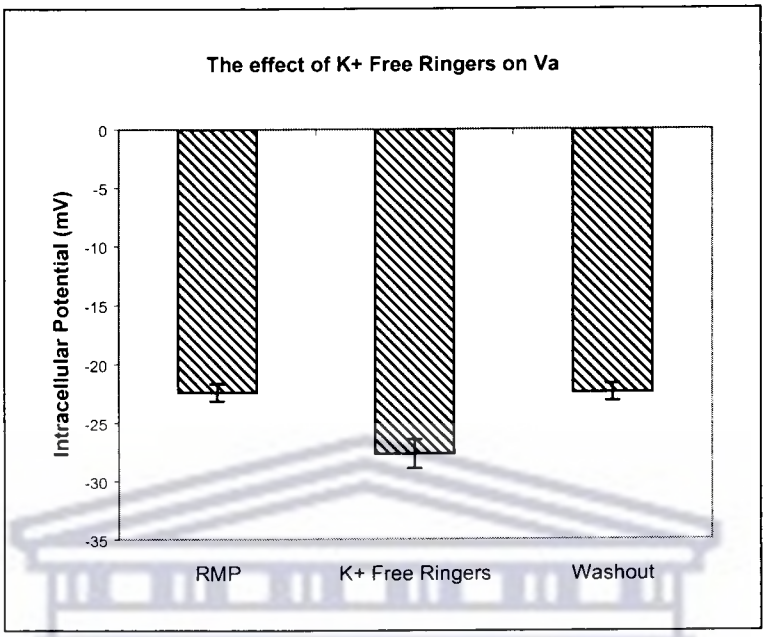
Under control conditions, Va and Vbl were –22.38±0.74 mV (N=3) and -47±0.41 mV (N=3) respectively. Removing the bath potassium caused a hyperpolarisation to –27.67±1.24 mV (P<0.013) in Va and –54.5±0.7 mV (P<0.0007) in Vbl. Upon the rinsing of potassium free Ringers with normal Ringers Va and Vbl returned to control values (P<1).

Theoretical Vt was calculated to be –24.3±1.03 mV under control conditions. The administration of K^+ free Ringers caused no significant change. Vt was calculated to be –26.83±1.31 mV (P<0.11) after the administration of K^+ free Ringers .

Table 4.5 The effect of K^+ free Ringers on potential

	RMP	**K^+ free Ringers**	**Washout**	**RMP vs K^+ Ringers**	**RMP vs Washout**
Va	-22.38±0.74	-27.67±1.24	-22.38±0.74	P<0.013	N.S
Vbl	-47±0.41	-54.5±0.7	-47±0.41	P<0.0007	N.S
Vt	–24.3±1.03	–26.83±1.31	–24.3±1.03	N.S	N.S

(A)



(B)

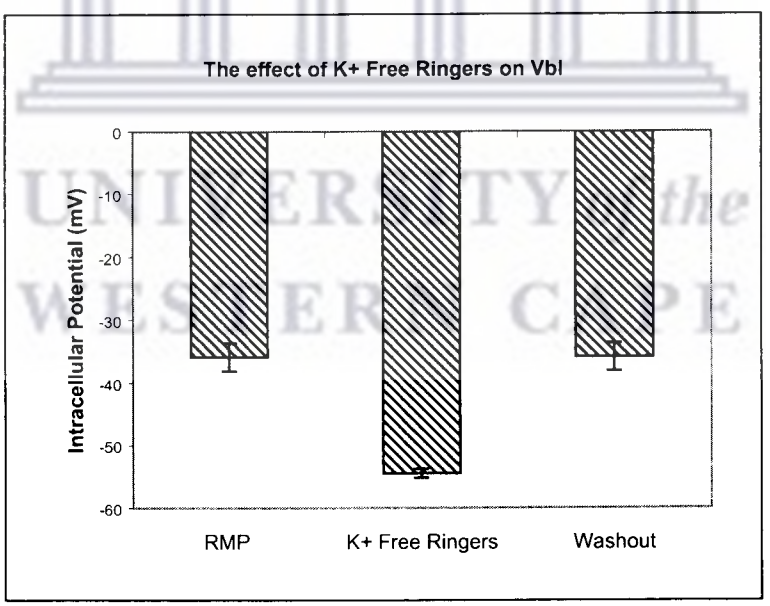


Figure 4.10 The effect of K free Ringers on (A) Va and (B) Vbl

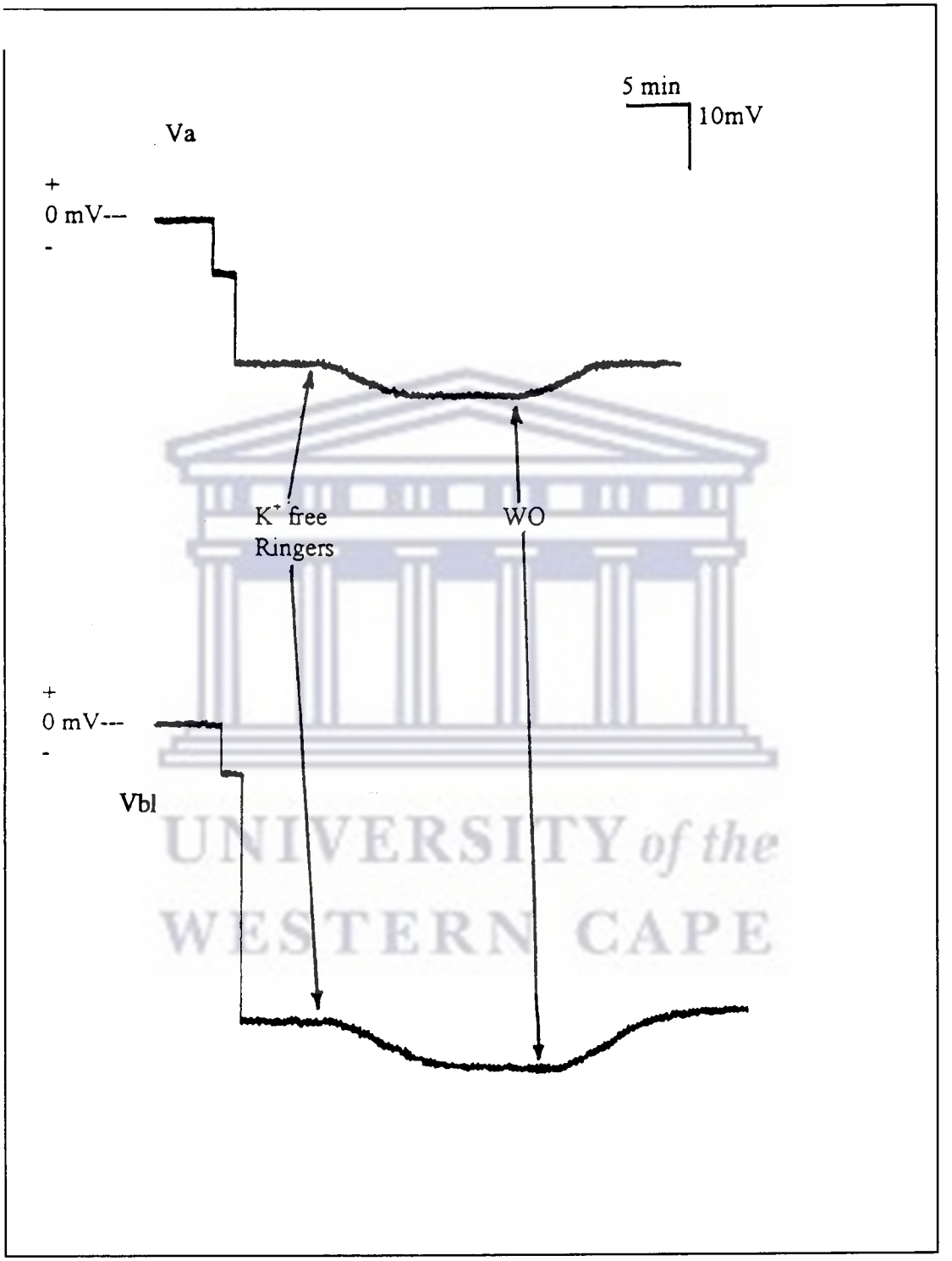


Figure 4.11 The effect of K free Ringers on Va and Vbl. WO=washout with bath Ringers.

4.9 Effects of Amiloride on the apical and basolateral membranes

Amiloride, a blocker of sodium channels (Ko *et al.*, 1998), was administered to the different bath fluids at a concentration of 10^{-6}M to test whether sodium channels exist within the apical and basolateral membranes of the TM4 Sertoli cell. The statistically analysed data are graphically represented in figure 4.12 and tabulated in table 4.6, while figure 4.13 illustrates a typical result as digitally recorded by the Clampex program during experimentation.

Under control conditions Va was –21.42±2.08 mV (N=6) in normal Ringers solution. The addition of amiloride caused an immediate hyperpolarization of all the potentials. In the presence of amiloride, Va was hyperpolarized to –39.92±0.74 mV (P<0.0001). Upon the replacement of amiloride with normal Ringers, Va recovered and returned to –21.92±2.44 mV (P<0.71).

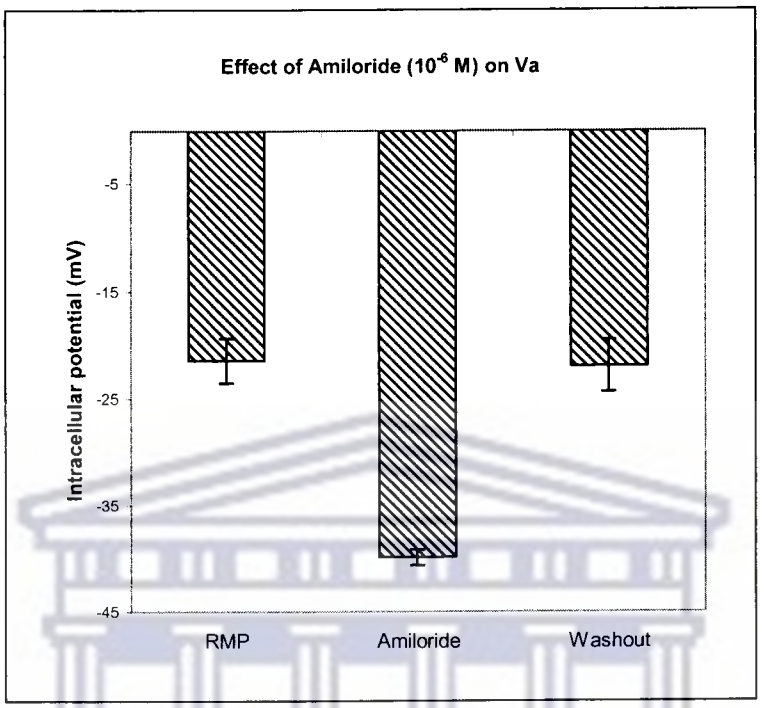
When the basolateral membrane was exposed to amiloride, Vbl hyperpolarized from the RMP of –47.92±0.97 mV to –59.5±0.89 mV (N=6) (P<0.0001). Upon rinsing with normal Ringers, Vbl returned to –48.17±2.01 mV (P<0.79).

Utilising the recorded Va and Vbl data, the theoretical transepithelial resistances (Vt) were calculated to be –26.5±1.04 mV at RMP, –19.58±0.73 (P<0.0001) mV after the administration of amiloride and –26.25±3.34 (P<0.88) mV after washout.

Table 4.6 ***Effect of amiloride on intracellular potential (mV). N.S = not significant***

	N	RMP	Amiloride	Washout	RMP vs amiloride	RMP vs WO
Va	6	–21.42±2.08	–39.92±0.74	–21.92±2.44	P<0.0001	N.S
Vbl	6	–47.92±0.97	–59.5±0.89	–48.17±2.01	P<0.0001	N.S
Vt	6	–26.5±1.04	–19.58±0.73	–26.25±3.34	P<0.0001	N.S

(A)



(B)

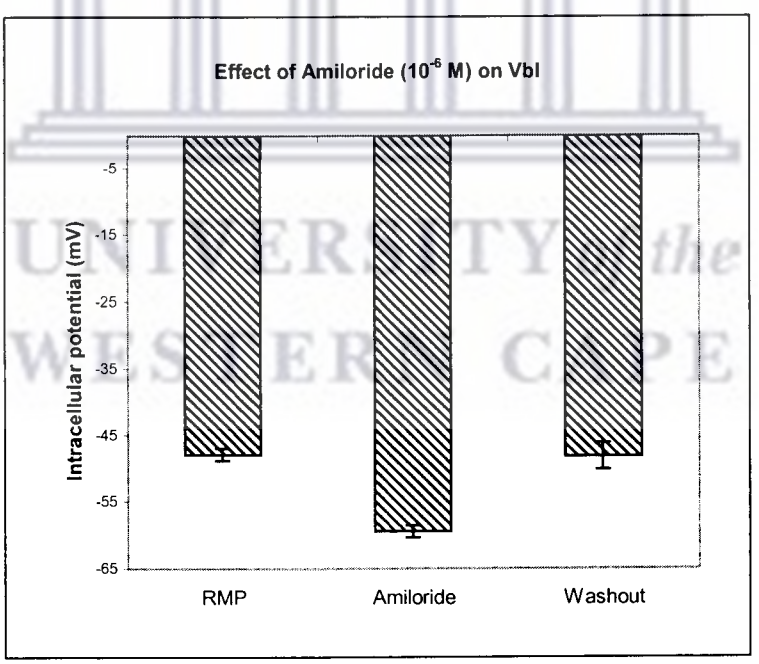


Figure 4.12 Graphs illustrating the effect of amiloride on (A) Va and (B) Vbl. WO = washout with normal Ringers.

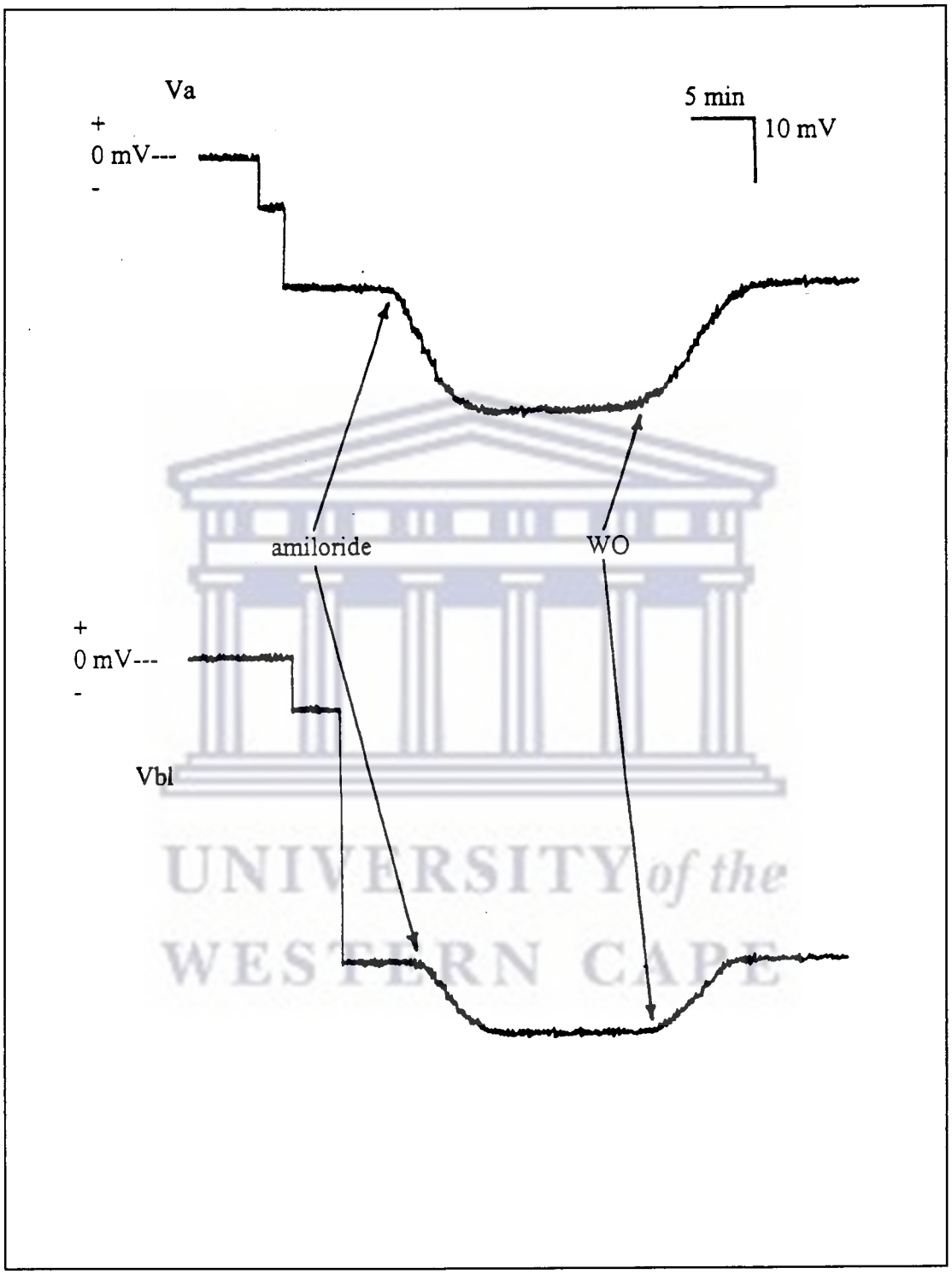


Figure 4.13 The effect of amiloride on Va and Vbl. WO=washout with bath Ringers.

4.10 The effect of Na^+ free Ringers

The effect of removing the bath medium's sodium concentration is summarised in table 4.7 and illustrated in figure 4.14, while figure 4.15 illustrates a typical result as digitally recorded by the Clampex program during experimentation. Under control conditions, Va and Vbl were –22.38±0.74 mV (N=3) and -47±0.41 mV (N=3) respectively.

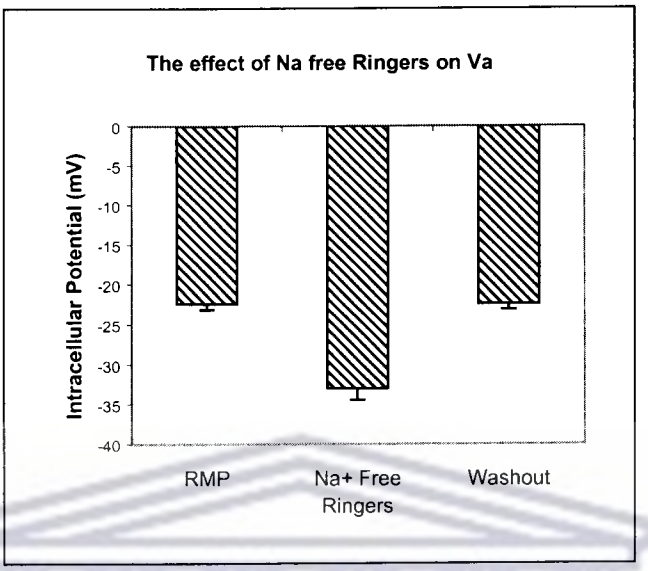
Removing the bath sodium caused a hyperpolarisation to -33±1.47 mV (P<0.004) in Va and –59.67±1.43 mV (P<0.003) in Vbl. Upon the rinsing of sodium free Ringers with normal Ringers Va and Vbl returned to control values (P<1).

Theoretical Vt was calculated to be –24.3±1.03 mV under control conditions. The administration of Na^+ free Ringers did not significantly change Vt. Vt was –26.67±1.02 mV (P<0.08). Upon washout Vt became –24.3±1.03 mV.

Table 4.7 The effect of Na^+ free Ringers on potential

	RMP	**Na^+ free Ringers**	**Washout**	**RMP vs Na^+ free**	**RMP vs Washout**
Va	-22.38±0.74	-33±1.47	-22.38±0.74	P<0.004	N.S
Vbl	-47±0.41	-59.67±1.43	-47±0.41	P<0.003	N.S
Vt	–24.3±1.03	–26.67±1.02	–24.3±1.03	P<0.08	N.S

(A)



(B)

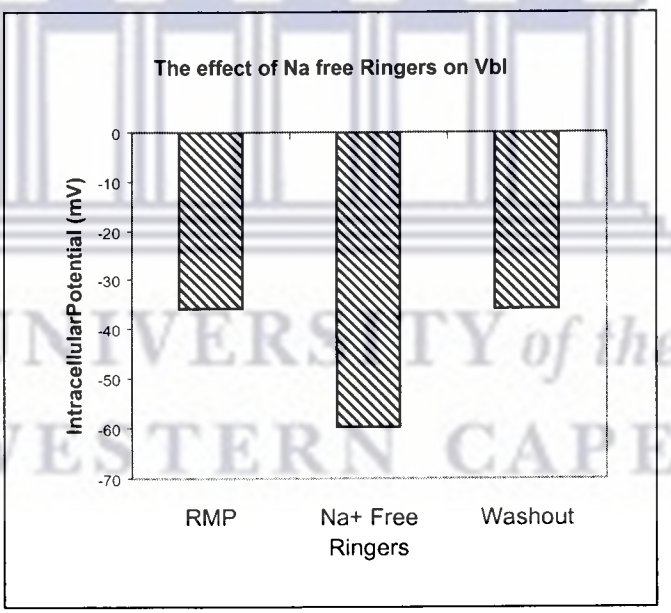


Figure 4.14 The effect of Na free Ringers on (A) Va and (B) Vbl

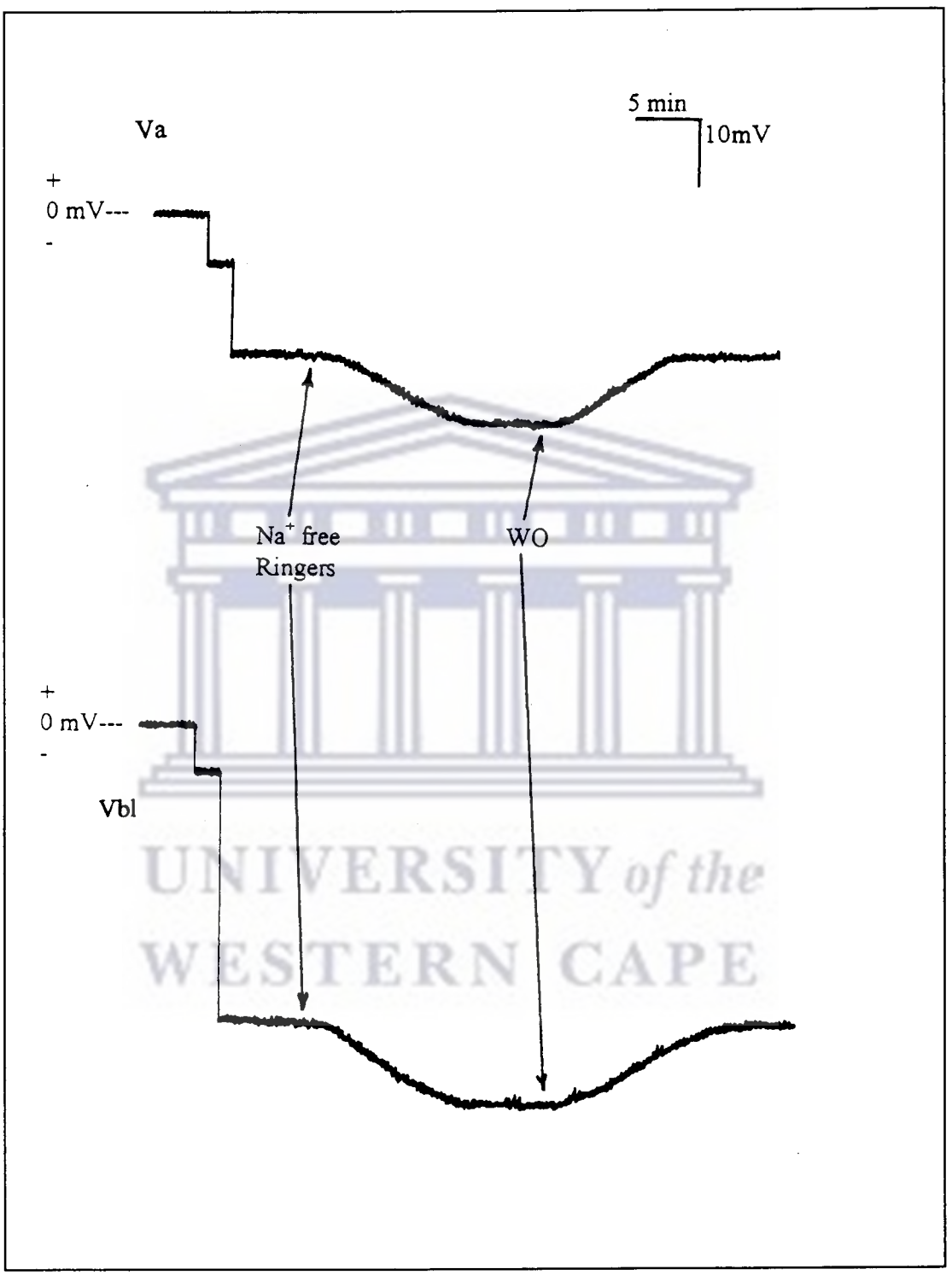


Figure 4.15 The effect of Na+ free Ringers on Va and Vbl. WO=washout with bath Ringers.

4.11 Effects of Acetazolamide on the apical and basolateral membranes

Acetazolamide, an inhibitor of carbonic anhydrase, was administered to both sides of the monolayer at a concentration of 10^{-6}M. This was done to test the hypothesis that generation of bicarbonate ions influences the Vi across TM4 Sertoli cell. The statistically analysed data are graphically represented in figure 4.16 and tabulated in table 4.8, while figure 4.17 illustrates a typical result as digitally recorded by the Clampex program during experimentation.

Under control conditions Va was –22±2.3 mV (N=6) in normal Ringers solution. The addition of acetazolamide caused an immediate depolarization of all the potentials. In the presence of acetazolamide, Va was depolarized to +13±1.52 mV (P<0.0001). On the replacement of acetazolamide Ringers with normal Ringers, Va recovered and returned to –21.42±2.08 mV (P<0.66).

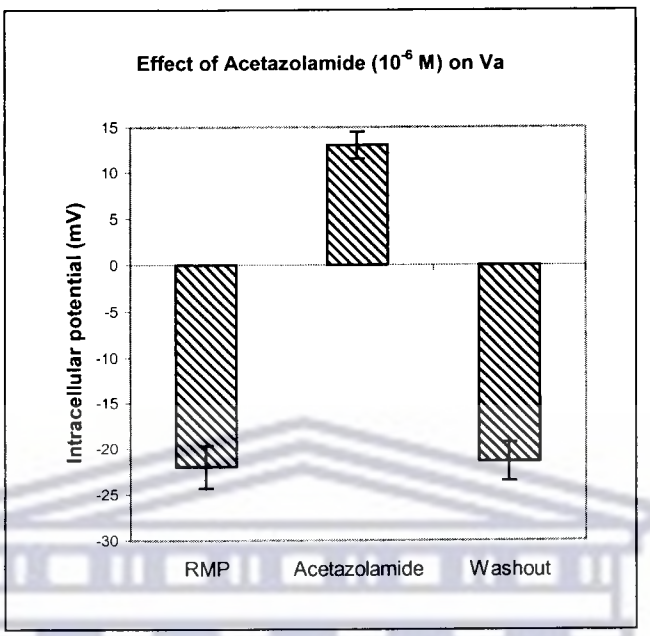
When tested on the basolateral membrane, Vbl depolarized from the RMP of –46.92±1.36 mV to –8.67±0.88 mV (N=6) (P<0.0001). Upon rinsing with normal Ringers, Vbl returned to –48.25±2.19 mV (P<0.24).

Utilising the recorded Va and Vbl data, the theoretical transepithelial resistances (Vt) were calculated to be –24.92±2.76 mV at RMP, –21.67±1.31 mV (P<0.05) after the administration of acetazolamide and –26.83±3.1 mV (P<0.32) after washout.

Table 4.8 ***Effect of acetazolamide on intracellular potential (mV).***

	N	RMP	Acetazolamide	Washout	RMP vs acetazolamide	RMP vs WO
Va	6	–22±2.3	+13±1.52	–21.42±2.1	P<0.0001	N.S
Vbl	6	–46.92±1.36	–8.67±0.88	–48.25±2.19	P<0.0001	N.S
Vt	6	–24.92±2.76	–21.67±1.31	–26.83±3.1	P<0.05	N.S

(A)



(B)

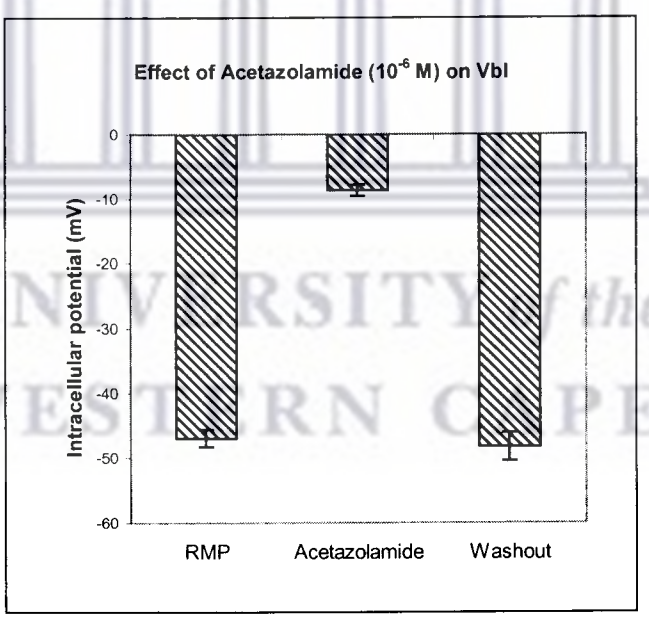


Figure 4.16 Graphs illustrating the effect of acetazolamide (10^{-6}M) on (A) Va and (B) Vbl.

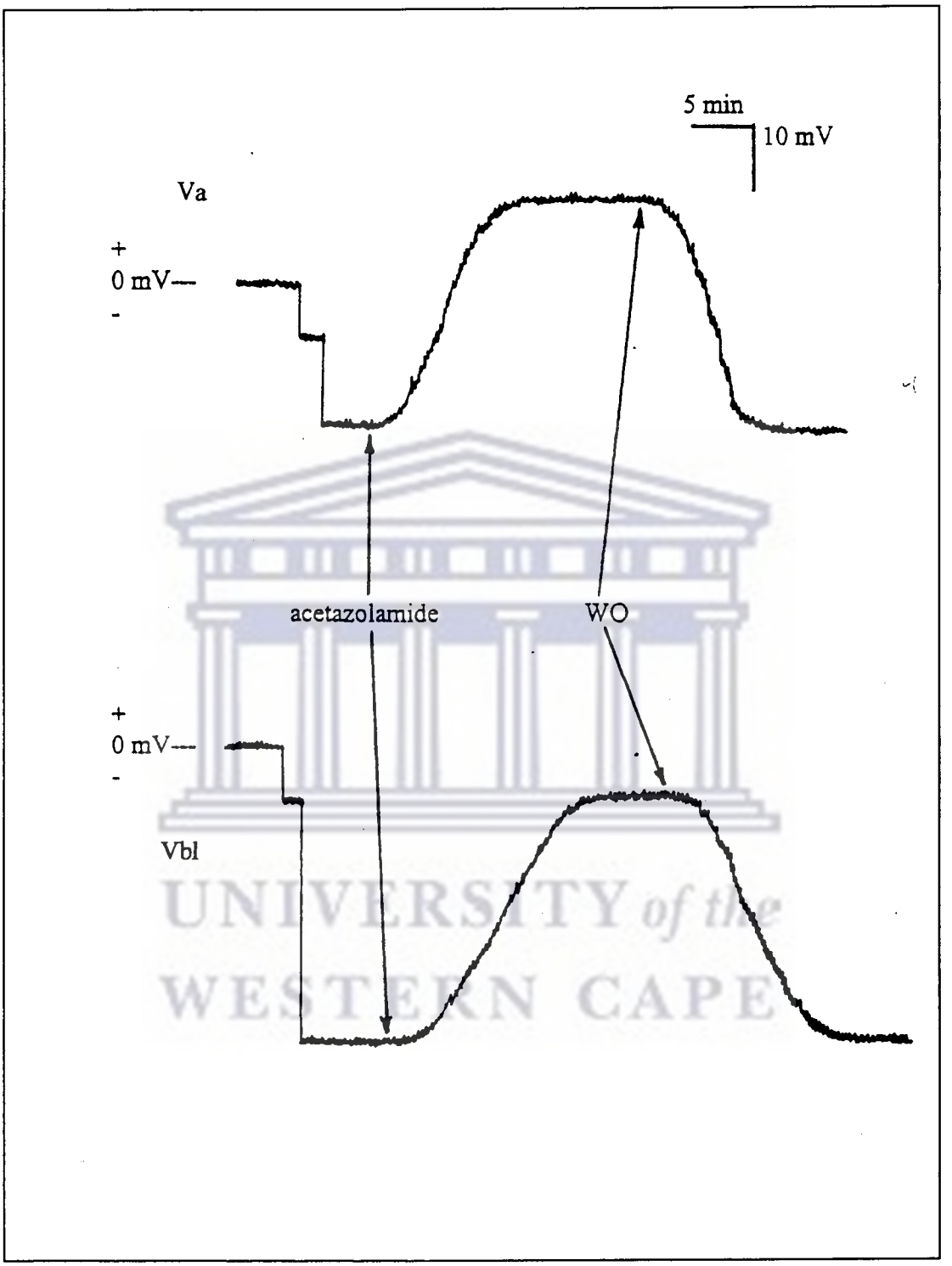


Figure 4.17 The effect of acetazolamide on Va and Vbl. WO=washout with bath Ringers.

4.12 The effect of different extracts on Rt

In an independent study different doses of extracts were administered after 48 hours of culture to observe any effects on Rt of the confluent monolayers of TM4 Sertoli cells. With a 24-hour incubation period these extracts were shown to cause a sharp decrease in Rt at all doses. Figure 4.18 best illustrates the response of the monolayers to different concentrations of extracts.

The controls measured at 371.1±70.2 $\Omega.cm^2$ after 24 hours. Cells that had undergone administration of the 50 μg/ml OA measured at 111.33±17.93 $\Omega.cm^2$, the 100 μg/ml treated monolayers had Rt of 115±18.55 $\Omega.cm^2$ and the 200 μg/ml treated monolayers developed a Rt of 108.33±19.6 $\Omega.cm^2$.

Non-parametric statistical analysis (Kruskal Wallis) showed that the OA treated groups all differed significantly from the control ($P<0.05$), but did not differ amongst each other.

Cells that had undergone treatment with extract from *Olea europaea* (OU) had Rt of 99±9.63 $\Omega.cm^2$, 98.3±14.7 $\Omega.cm^2$ and 94.7±14.4 $\Omega.cm^2$ for 50 μg/ml, 100 μg/ml and 200 μg/ml respectively. Statistical analysis showed the difference between the control and the treated groups ($P<0.03$), but no significant difference between the treatments.

Cells that underwent treatment with extract from *Olea exasperata* (OX) had Rt of 106±15.3 $\Omega.cm^2$, 110±15.3 $\Omega.cm^2$ and 98.7±15.2 $\Omega.cm^2$ for 50 μg/ml, 100 μg/ml and 200 μg/ml respectively after the 24-hour exposure to this extract. Statistical analysis showed the difference between the control and the treated groups ($P<0.05$), but no significant difference between the treatments.

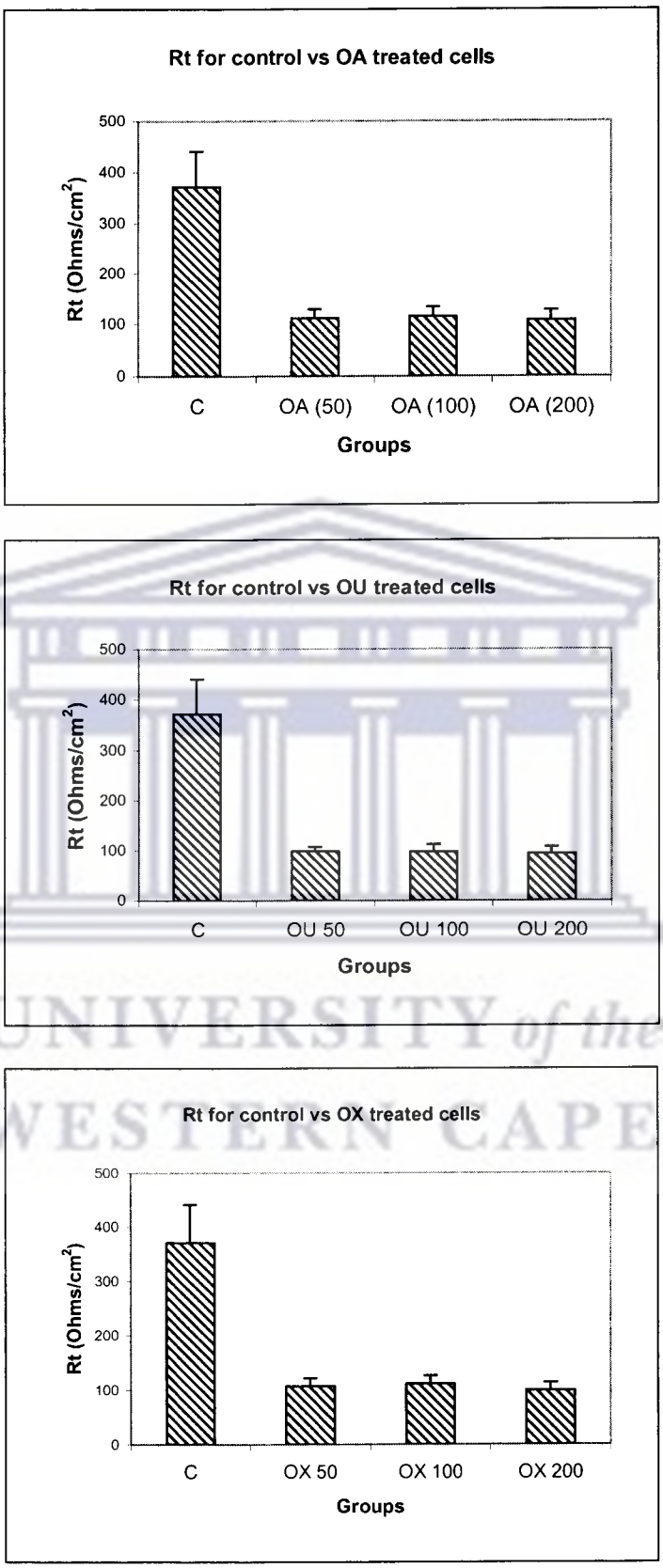


Figure 4.18 Graphs of the effect of different doses of extracts on Rt. (A) Control vs OA, (B) Control vs OU and (C) Control vs OX.

4.13 Effects of oleanolic acid on Va and Vbl

Cells that underwent a 24-hour treatment of OA were studied to determine whether they had undergone significant change in comparison to the control. Monitoring the intracellular potential of the treated cells with that of the control and establishing whether the potential had varied with the administration of the different doses of extract would assess these comparisons.

Va for cells that underwent treatment with 50 μg/ml, 100 μg/ml and 200 μg/ml had potentials of –21.67±1.94 mV (N=6), –21.42±2.08 mV (N=6) and –22.42±2.11 mV (N=6). Statistical analysis using Kruskal-Wallis variance analysis revealed no significant differences between these groups and the control (P<0.81).

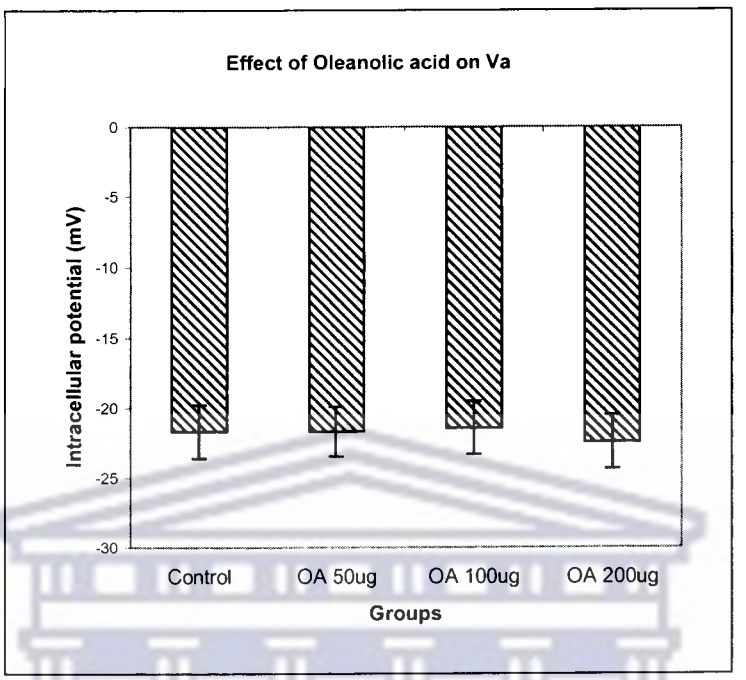
Vbl for these cells was determined to be –48.08±1.77 mV (N=6), –47.92±2.22 mV (N=6) and –47.67±2.11 mV (N=6) for treatments of 50 μg/ml, 100 μg/ml and 200 μg/ml respectively (P<0.97). Figure 4.3 illustrates the comparison of treated cells with the control.

Vt was determined to be –27±3.51 mV for the control, –26.42±3.05 mV at 50 μg/ml (N=6), –26.5±3.51 mV at 100 μg/ml (N=6) and –25.25±1.65 mV (N=6) at 200 μg/ml (P<0.77).

Table 4.9 ***Comparative potentials (in mV) for OA treated cells***

	Va	**Vbl**	**Vt**
Control	–21.67±1.91	–47.46±1.74	–27±3.51
OA (50 μg/ml)	–21.67±1.94	–48.08±1.77	–26.42±3.05
OA (100 μg/ml)	–21.42±2.08	–47.92±2.22	–26.5±3.51
OA (200 μg/ml)	–22.42±2.11	–47.67±2.11	–25.25±1.65

(A)



(B)

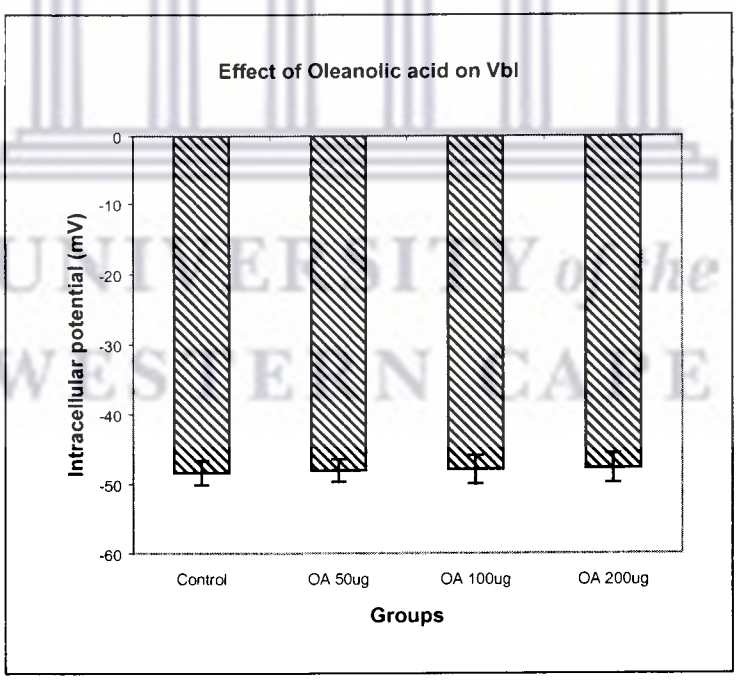


Figure 4.19 The effect of oleanolic acid (OA) on (A) Va and (B) Vbl

4.14 Effect of crude extract from Olea europaea (OU) on Va and Vbl

Cells that underwent a 24-hour treatment of OU were studied to determine whether they had undergone significant change in comparison to the control. Monitoring the intracellular potential of the treated cells with that of the control and establishing whether the potential had varied with the administration of the different doses of extract would assess these comparisons.

Va for cells that underwent treatment with 50 μg/ml, 100 μg/ml and 200 μg/ml had potentials of -21.83±2.16 mV (N=6), -21.33±1.91 mV (N=6) and -22.4±2.1 mV (N=6). Statistical analysis using Kruskal-Wallis variance analysis revealed no significant differences between these groups and the control ($P<0.8$).

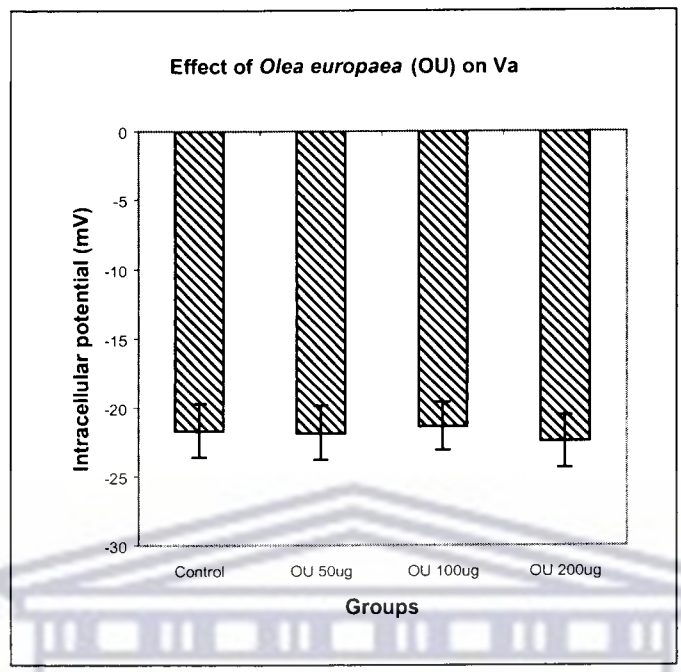
Vbl for these cells was determined to be -48.08±1.77 mV (N=6), -47.92±2.22 mV (N=6) and -47.67±2.29 mV (N=6) for treatments of 50 μg/ml, 100 μg/ml and 200 μg/ml respectively ($P<0.97$). Figure 4.20 illustrates the comparison of treated cells with the control.

Vt was determined to be –27±3.51 mV for the control, –26.42±3.05 mV at 50 μg/ml (N=6), –26.5±3.51 mV at 100 μg/ml (N=6) and –25.25±1.65 mV (N=6) at 200 μg/ml ($P<0.95$).

Table 4.10 ***Comparative potentials (in mV) for OU treated cells***

	Va	**Vbl**	**Vt**
Control	–21.67±1.91	–47.46±1.74	–27±3.51
OU (50 μg/ml)	–21.83±2.16	–48.08±1.77	–26.25±3.24
OU (100 μg/ml)	–21.33±1.91	–47.92±2.22	–26.58±3.37
OU (200 μg/ml)	–22.4±2.1	–47.67±2.29	–25.25±1.65

(A)



(B)

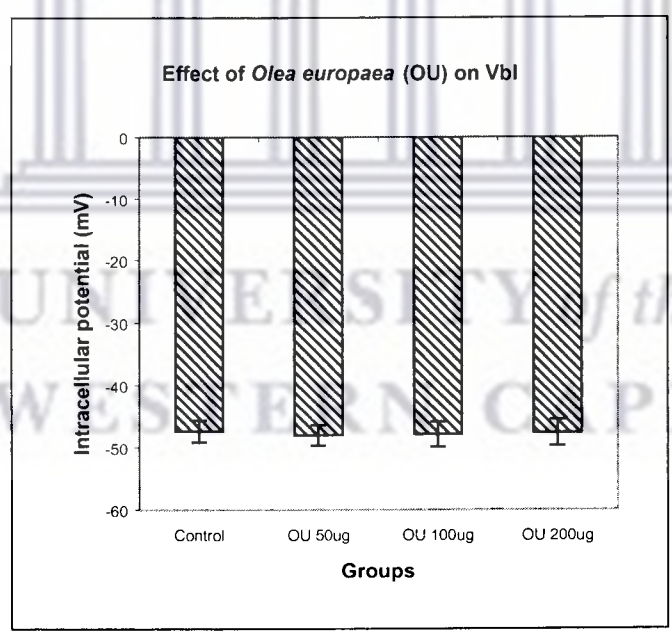


Figure 4.20 The effect of Olea europaea (OU) on (A) Va and (B) Vbl

4.15 Effect of crude extract from Olea exasperata (OX) on Va and Vbl

Cells that underwent a 24-hour treatment of OX were studied to determine whether they had undergone significant change in comparison to the control. Monitoring the intracellular potential of the treated cells with that of the control and establishing whether the potential had varied with the administration of the different doses of extract would assess these comparisons.

Va for cells that underwent treatment with 50 μg/ml, 100 μg/ml and 200 μg/ml had potentials of –21.58±2.08 mV (N=6), -21.75±2.12 mV (N=6) and –22.42±2.02 mV (N=6). Statistical analysis using Kruskal-Wallis variance analysis revealed no significant differences between these groups and the control (P<0.88).

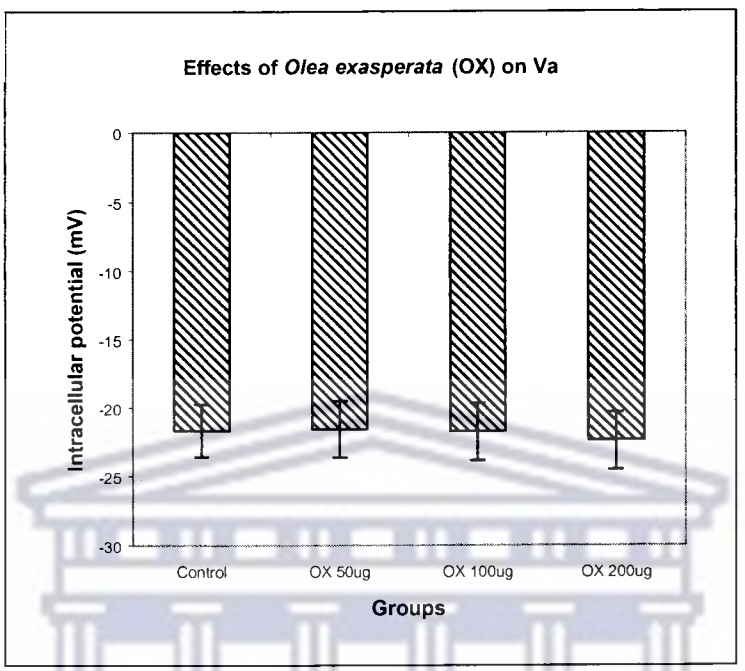
Vbl for these cells was determined to be –47.58±2.08 mV (N=6), -47.83±1.63 mV (N=6) and –47.92±1.77 mV (N=6) for treatments of 50 μg/ml, 100 μg/ml and 200 μg/ml respectively (P<0.79). Figure 4.22 illustrates the comparison of treated cells with the control.

Vt was determined to be –27±3.51 mV for the control, –26±1.15 mV at 50 μg/ml (N=6), –26.08±1.59 mV at 100 μg/ml (N=6) and –25.5±1.73 mV (N=6) at 200 μg/ml (P<0.88).

Table 4.11 ***Comparative potentials (in mV) for OX treated cells***

	Va	Vbl	Vt
Control	–21.67±1.91	–47.46±1.74	–27±3.51
OX (50 μg/ml)	–21.58±2.08	–47.58±2.08	–26±1.15
OX (100 μg/ml)	–21.75±2.12	–47.83±1.63	–26.08±1.59
OX (200 μg/ml)	–22.42±2.02	–47.92±1.77	–25.5±1.73

(A)



(B)

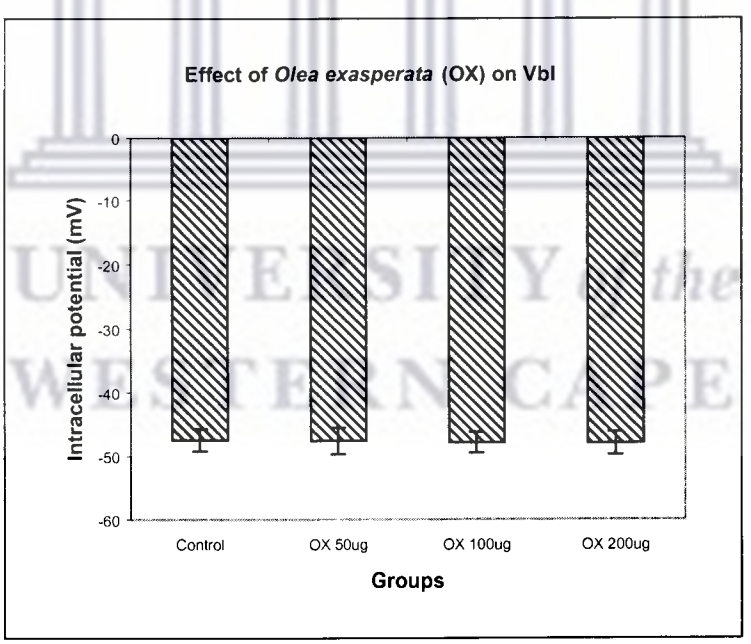


Figure 4.21 The effect of OX on (A) Va and (B) Vbl

Table 4.12 Control and experimental values.

A SUMMARY OF THE EFFECTS ON THE APICAL MEMBRANE POTENTIAL				
BATH RINGERS	N	CONTROL	EXPERIMENT	STATISTICS
Furosemide	6	-21.08±1.28	-6±0.71	$P<0.0001$
Bumetanide	6	-22±2.3	-11.92±1.02	$P<0.0001$
Acetazolamide	6	-22±2.3	+13±1.52	$P<0.0001$
Amiloride	6	-21.42±2.08	-39.92±0.74	$P<0.0001$
$BaCl_2$	5	-20.5±1.73	-20.5±1.73	N.S
CFR	4	-22.38±0.74	-7.5±1.62	$P<0.0001$
Na^+ free Ringers	3	-22.38±0.74	-33±1.47	$P<0.004$
K^+ free Ringers	3	-22.38±0.74	-27.67±1.24	$P<0.013$
OA*	18	-21.67±1.91	-21.73±1.96	N.S
OU*	18	-21.67±1.91	-21.98±2	N.S
OX*	18	-21.67±1.91	-21.8±1.98	N.S

Values expressed as means in millivolts (mV). N is the number of cells; N.S., not significant; paired T-tests were used unless marked with * where non-parametric analysis of variance (Kruskal-Wallis) used. $BaCl_2$ - Barium chloride (2mM); CFR – chloride free Ringers; OA – oleanolic acid; OU – *Olea europaea* extract; OX – *Olea exasperata* extract.

Table 4.13 Control and experimental values.

A SUMMARY OF THE EFFECTS ON THE BASOLATERAL MEMBRANE POTENTIAL				
BATH RINGERS	N	CONTROL	EXPERIMENT	STATISTICS
Furosemide	6	-46.3±0.93	-27.08±0.73	$P<0.0001$
Bumetanide	6	-48.42±1.88	-39.17±1.37	$P<0.0001$
Acetazolamide	6	-46.92±1.36	-8.67±0.88	$P<0.0001$
Amiloride	6	-47.92±0.97	-59.5±0.89	$P<0.0001$
$BaCl_2$	5	-45.5±1.89	-45.5±1.89	N.S
CFR	4	-47±0.41	-35.8±2.25	$P<0.002$
Na^+ free Ringers	3	-47±0.41	-59.67±1.43	$P<0.003$
K^+ free Ringers	3	-47±0.41	-54.5±0.7	$P<0.0007$
OA*	18	-47.46±1.74	-48.02±1.97	N.S
OU*	18	-47.46±1.74	-48.02±1.94	N.S
OX*	18	-47.46±1.74	-47.98±1.75	N.S

Values expressed as means in millivolts (mV). N is the number of cells; N.S., not significant; paired T-tests were used unless marked with * where non-parametric analysis of variance (Kruskal-Wallis) used. $BaCl_2$ - Barium chloride (2mM); CFR – chloride free Ringers; OA – oleanolic acid; OU – *Olea europaea* extract; OX – *Olea exasperata* extract.

CHAPTER 5

DISCUSSION

5.1 Introduction

In this chapter the results (mainly chapter 4) will be discussed in detail. The chapter concludes with a summary of the main findings of this study. Although much data exists for primary cultures using rat Sertoli cells, no known literature on the TM4 Sertoli cell (mouse) grown in bicameral chambers to our knowledge has been published in the scientific literature. Thus the data presented here is novel and similarities and contrasts with studies using primary rat cultures will be drawn.

5.2 The measurement of transepithelial resistances

5.2.1 Controls

5.2.1.1 Effects of different seeding concentrations on Rt

In order to gauge the optimum amount of TM4 Sertoli cells seeded on matrigel-coated Millipore filters, two different concentrations were seeded and observed over a 6-day period, during which Rt was measured on a daily basis. Our study shows that 10^5 cells per filter could be effectively grown for up to 6 days while maintaining a relatively constant Rt.

After 3 days cells seeded at 10^6 cells per well peeled off the matrigel-coated Millipore filter surface as observed under an inverted microscope. The decrease in Rt from day four onwards indicated that the layer of cells immediately adjacent to the filter surface had also peeled off, resulting in the sharp and significant ($P<0.0001$) decrease in Rt.

Cells seeded at 10^5 cells per well appear to develop multilayers without compromising their ability to adhere to the matrigel-coated Millipore filter surface. This was endorsed by histological studies (see next section).

Another interesting aspect of this study was that cells formed a high resistance barrier after 24 hours. This indicated the formation of tight junctions within this layer. This contrasts with primary rat cells in that Rt progressively increased to plateau and stabilise between three and five days (Janecki *et al.*, 1991). Primary rat Sertoli cells develops Rt stability between days four and five, whereafter there is a slight and insignificant decrease in Rt. This trend was also seen in the current study (see fig 4.1).

Our studies show a significantly lower Rt for the same culturing protocol but remarkably similar to the Rt achieved for primary rat cultures that did not include FSH. This appears to indicate that FSH does not have the same effects on TM4 cells as with primary rat cell cultures with respect to monolayer resistance (Janecki *et al.*, 1991).

5.2.1.2 The effects of supplemented media on the histology of TM4 Sertoli cells

This study showed that cultured TM4 Sertoli cells have a cuboidal shape when cultured in the presence of serums and hormones and that the same cells grown in unsupplemented media resemble squamous epithelia (Results, section 4.3).

In comparison to studies performed by Onoda *et al.* (1990), it can also be deduced that the columnar-like Sertoli primary rat cells cultured by these researchers differ in shape to the cuboidal-like TM4 Sertoli cell cultures achieved in this study. Another interesting difference is that TM4 Sertoli cells develop multilayers within 3 days whereas only single layers have been reported for primary rat cultures (Onoda *et al.*, 1990). Again, it is important to keep in mind that the TM4 Sertoli cell is an immortalised mouse cell line.

This study has highlighted the importance of using supplemented media for studies where confluency is a requirement and has revealed a number of differences and similarities between TM4 and primary rat cells grown in a bicameral system. These are summarised in table 5.1.

Table 5.1 The differences and similarities of TM4 cells and primary rat cells

Differences	*Similarities*
1) TM4 cells form multilayers while primary rat cultures form only single layers of cells (Onoda *et al.*, 1990).	1) Rt dependant on media supplementation (Janecki *et al.*, 1991)
2) TM4 cells have cuboidal shape, while primary rat cells have columnar shape (Onoda *et al.*, 1990).	2) Once Rt attains maximum levels, similar trends of slightly insignificant decreases occur in Rt.
3) After 24 hours TM4 cells have maximum Rt. For primary rat cultures the maximum Rt develops after 4-5 days (Janecki *et al.*, 1990).	

5.2.1.3 Control membrane potentials

It is well established in electrophysiological literature (Baldrick *et al.*, 1988) that the potential across a single layer epithelium, transepithelial potential (Vt) is closely approximated by the sum of the series potentials of that across the apical and basolateral membranes. Based on the above, the theoretical Vt was found to be -25.49±2.49 mV (N=36).

This potential is analogous to the Vt (luminal potential) of the seminiferous tubule. However, both the *in vivo* (Tuck *et al.*, 1970 and Cuthbert and Wong, 1975) and the *in vitro* (Fisher, 2002) studies have indicated the luminal potential to be in the region of –4 to –6 mV.

As this is the first study to measure both Va and Vbl in a bicameral system using TM4 Sertoli cells, we could only compare our data with intracellular studies carried out on seminiferous tubules. In this case, our Vbl data represents most closely the intracellular potential (Vi) data obtained across the basolateral membranes of the seminiferous tubule.

Vbl was found to be significantly more negative in TM4 cell cultures. However, the literature has a wide spread of values which range between –21.6 mV (Roche and Joffre, 1989) for cultured cells to –37.65 mV for Sertoli cell enriched (only) seminiferous tubules (Wasserman *et al.*, 1992).

5.3 The effect of extracts

5.3.1 Effects on Rt

Crude extracts for both *Olea europaea* and *Olea exasperata* have shown to possess copious quantities of oleanolic acid (Garcia-Granados *et al.*, 1998). The effects on Rt by these extracts showed no significant difference to that of purified OA. However, our data did not exhibit a gradual dose effect. The reason for this is that the concentration used in this study produced a maximal response and therefore probably masked the dose response. We suspect that lower doses of OA would show a dose response curve.

The absence of the dose response does not defer from the significance of OA on Rt. After 24 hours, OA produced a sharp and significant decline when compared to the control monolayers. The reason for this decrease in Rt across the monolayer appears to be limited and located to the level of the intercellular junctions.

This line of reasoning is endorsed by the histological studies carried out on mice (see chapter 2) treated with daily intraperitoneal doses of OA. The histology of the testes showed a breakdown in the germinal epithelium, which appear to be located primarily at the level of the tight junctions.

It is interesting to note that all published studies on rodents have shown a complete reversal of infertility (Rajasekaran *et al.*, 1988) subsequent to the cessation of OA treatment (Mdhluli and Van der Horst, 2002).

5.3.2 The effects on Vi

The administration of different doses of extracts were shown to have no effect on intracellular potential. Oleanolic acid did not alter the intracellular potential of either Va or Vbl, showing no significant differences in comparison to the controls. Similarly, crude extracts from *Olea europaea* and *Olea exasperata* showed no effects on Va or Vbl after a 24-hour administration period.

The fact that OA does not change the resting membrane potential of TM4 cells seems to indicate that it only interferes with mechanisms involved in the establishment of intercellular junctions.

This further demonstrates our hypothesis that OA affects the breakdown of the intercellular junctions, while leaving the Sertoli cell intact and viable. Thus, after the cessation of OA treatment, Sertoli cells are able to regenerate the intercellular junctions and establish the blood-testis barrier in order for the process of spermatogenesis to resume.

5.4 Exploring the basic electrophysiological properties of the TM4 Sertoli cell

5.4.1 Introduction

Although much electrophysiological studies have been performed on the cultured primary rat Sertoli cell, little is known about the ionic transport properties of the TM4 Sertoli cell grown on a matrigel-coated Millipore filter.

This study attempts to characterise both the apical and basolateral membranes of the TM4 cell electrophysiologically for the first time.

5.4.2 Examining chloride transport

One of the only electrophysiological studies of TM4 cells (Jungwirth *et al.*, 1997) has clearly implicated the role of chloride in the response to FSH. However, the underlining mechanisms have still to be elucidated.

5.4.2.1 Effects of bumetanide

In order to characterise the chloride transport in the TM4 cell, bumetanide, a well-known blocker of $Na^+K^+2Cl^-$ co-transport (Ko *et al.*, 1998) was administered to the apical and basal media of confluent TM4 monolayers during intracellular recording of resting membrane potential.

Bumetanide exposure depolarised both the apical and basolateral membranes. This indicated that on both membranes mechanisms for the co-transport of chloride ions is present.

The ease at which the depolarisation was reversed, subsequent to the removal of bumetanide from the Ringers, suggest the mechanism whereby this inhibitor blocks membrane co-transport of chloride to be located primarily at the level of the membrane, and thus, it is unlikely that this mechanism involves cytoplasmic mechanisms.

This is the only study which investigated the effects of bumetanide on the resting membrane potentials of TM4 cells. Only Ko *et al.* (1998) examined the effect of bumetanide on the patch clamp recorded short circuit current of primary rat cultured cells. Here, a decrease in the short circuit current was reported, supporting our results, which also indicated a decrease in the chloride transport across the Sertoli cell membrane.

5.4.2.2 Effects of furosemide

To substantiate the findings of bumetanide in this study, furosemide, another well-known $Na^+K^+2Cl^-$ co-transport blocker (Baldrick *et al.*, 1988), was administered to both the apical and basal media of the monolayer. The administration of furosemide to the monolayer caused a significant and even greater depolarisation in Vi, confirming the results attained with bumetanide administration. It is interesting to note, however, that the resultant furosemide induced depolarisation was greater than that of bumetanide. This suggests that the TM4 Sertoli cell is more sensitive to furosemide than bumetanide.

No comparative studies using furosemide on testicular tissue and cultured cells of the testes were found after extensive literature searches. Comparisons with the Malpighian tubules of the locust, however, contrasted sharply with the present study in that both Va and Vbl hyperpolarized and that the hyperpolarisation occurred relatively slower (12mV within 10 minutes) when compared to TM4 cells (approximately 6 minutes to reach a plateau of depolarisation (i.e. for both Va and Vbl)).

5.4.2.3 The effects of chloride free Ringers

Chloride free Ringers was introduced to both the basal and apical media to investigate the effects of removing chloride from the media on the resting membrane potential.

The introduction of chloride free Ringers caused an immediate depolarisation in both Va and Vbl, the sensitivity of the Vi to the change in the bath media an indication that both the apical and basolateral membranes of the TM4 Sertoli cell are freely permeable to chloride ions.

The depolarisation due to the ionic imbalance in the Ringers was noted to be more similar to the results shown with the introduction of furosemide than to bumetanide, suggesting

that furosemide is an optimal indicator of the monitoring the chloride transport mechanisms in the TM4 Sertoli cell.

5.4.3 The effect of $BaCl_2$ and K^+ free Ringers on Vi

It is well known that the luminal fluid of the seminiferous tubule has an unusually high concentration of potassium ions (40 – 60 mM). However, the mechanism whereby the transport of K^+ across the blood-testis barrier occurs, i.e. in the Sertoli cell, is largely unknown.

Upon the removal of K^+ from the extracellular media, a significant hyperpolarisation of the intracellular potential occurred. This is indicative that K^+ channels exist in the apical and basolateral membranes of the Sertoli cell. The hyperpolarisation probably resulted due to the absence of the dominant intracellular ion from the bathing media, namely potassium.

The Sertoli cell cultures were treated with 2mM $BaCl_2$, a blocker of K^+ channels (Weltens *et al.*, 1992), to test for the presence of these channels in the basolateral and apical membranes. Literature reports have given evidence to the existence of K^+ channels on the apical membrane (Waites and Gladwell, 1982) and that K^+ co-transport occurs at the basolateral membrane. TM4 Sertoli cells were unresponsive to $BaCl_2$. This, however, does not mean that there exist no potassium channels in the TM4 Sertoli cell membrane.

This suggests that barium sensitive channels do not exist in the TM4 cell. Other K^+ channel blockers may affect the K^+ channels and further studies in this respect need to be conducted. This line of thinking was supported by the effects of potassium removal from the bathing media and endorses the data in the literature (Jegou, 1992).

5.4.4 Examining sodium transport

It is widely accepted that the regulation of intracellular potential is directly linked to the amount of Na^+ pumped out of the cell. The mechanism whereby this occurs has been postulated, but in TM4 cells has not been proven electrophysiologically or otherwise. A study was, therefore, carried out with the intent of characterising the dependency of the intracellular potential of the TM4 Sertoli cell on Na^+ ions.

In the current study, hyperpolarisation was observed in response to amiloride administration of both Va and Vbl. The effect of amiloride on intracellular potential has not been previously reported on in the literature.

In a set of complementary experiments, the effect of sodium free Ringers was tested on the intracellular potential. Exposure of the apical and basolateral membranes to sodium free Ringers also resulted in hyperpolarisation of both membranes.

The latter result has endorsed previous findings where hyperpolarisation was also reported for the removal of Na^+ from the bathing media of seminiferous tubules, which were punctured with microelectrodes (Cuthbert and Wong, 1975). Our data showed that Va hyperpolarised by 18mV and Vbl hyperpolarized by 12 mV, whereas Cuthbert and Wong (1975) found that intracellular potential of the rat seminiferous tubule hyperpolarized by 3mV.

In our study, TM4 cells exhibit a greater response (hyperpolarisation) in Na^+ free media in comparison to the findings of Cuthbert and Wong (1975). Thus the current study endorses the view that both the apical and basolateral membranes are permeable to Na^+ ions. The fact that amiloride induced a response indicates that mechanisms for Na^+ H^+ exchangers or sodium channels exist in both membranes of the TM4 Sertoli cell.

5.4.5 Examining the effects of blocking carbonic anhydraze

In the current study, a drastic and significant depolarisation of Va and Vbl was observed in response to acetazolamide administration. This is the first study to measure the effect of this carbonic anhydraze blocker in TM4 cells. Cuthbert and Wong (1975) previously demonstrated that similar results while measuring the Vi of rat seminiferous tubules. A comparison to these findings was thus made.

In the current study Va and Vbl depolarised by approximately 35mV, in comparison to the depolarisation of 10mV in rat seminiferous tubules (Cuthbert and Wong, 1975).

This study has confirmed the findings in the literature (Cuthbert and Wong, 1975), where in the seminiferous tubules of rats the administration of acetazolamide caused the intracellular potentials to depolarise significantly. Va and Vbl depolarised from RMP, confirming that the absence of intracellular H_2CO_3 formation resulted in a loss of HCO_3^-, which led to the depolarisation of Va and Vbl.

Cuthbert and Wong (1975) established that acetazolamide causes a large and reversible depolarisation in membrane potential due to the reduction in the availability of intracellular bicarbonate. Our data for TM4 cells has shown a similar trend, however, in an increased sensitivity of carbonic anhydraze to acetazolamide exposure.

Thus HCO_3^- plays an important role in the maintenance of the resting membrane potential of the TM4 Sertoli cell.

5.5 General conclusion

The data generated in this study have produced novel data with regard to the membrane transport mechanisms of TM4 Sertoli cells. The study has demonstrated new control resting membrane potentials for the apical and basolateral membranes of the TM4 Sertoli cell. In addition, the study has verified the existence of a membrane transport mechanism

for Cl- transport, a possible Na^+H^+ exchanger and the existence of Na^+ and K^+ channels as declared in the literature. These findings have allowed us to characterise the TM4 Sertoli cell membranes with regard to these ionic transporters and have also indicated the degree of sensitivity to known ionic transport inhibitors. The study has also shown that there appears to be differences as to the membrane transport characteristics of TM4 cells compared to cells grown from primary cultures. This may be to the suppression of gene expression in immortalised cell line, like the TM4 cell line.

The data has further allowed for the presentation of a simplified electrical equivalent circuit (figure 5.1) for TM4 cells in a monolayer. The main features of this model comprise a set of resistivities (Ra and Rbl) and electric motor forces (Ea and Ebl), which reside at the apical and basolateral membranes. The measured Vt was close to 0mV and this verified data in the literature for monolayers. However, the control theoretical Vt was shown to be considerably higher. We are unsure currently to provide an explanation for this disparity.

Most importantly, this study has significantly provided answers as to the physiological mechanism whereby oleanolic acid brings about contraception in male mice and rats. The decrease in Rt across monolayers of Sertoli cells in conjunction with intracellular potentials remaining unchanged during OA exposure indicates strongly that OA acts via the intercellular junctional complexes, while leaving the Sertoli cell viable. The microscopy study (chapter 2) supports the view that the Sertoli cell tight junctions between adjacent Sertoli cells are impaired, which leads to a breakdown in the process of spermatogenesis.

The fact that OA does not destroy the integrity of the Sertoli cell and spermatogonia presents OA potentially as one of the most suitable candidates for male contraception. Added to this, its anti-ulcer, antimicrobial, anti-inflammatory, anti-tumor and anti-hyperlipidemia effects as well as its non-toxicity contributes to its attractiveness as a male contraceptive.

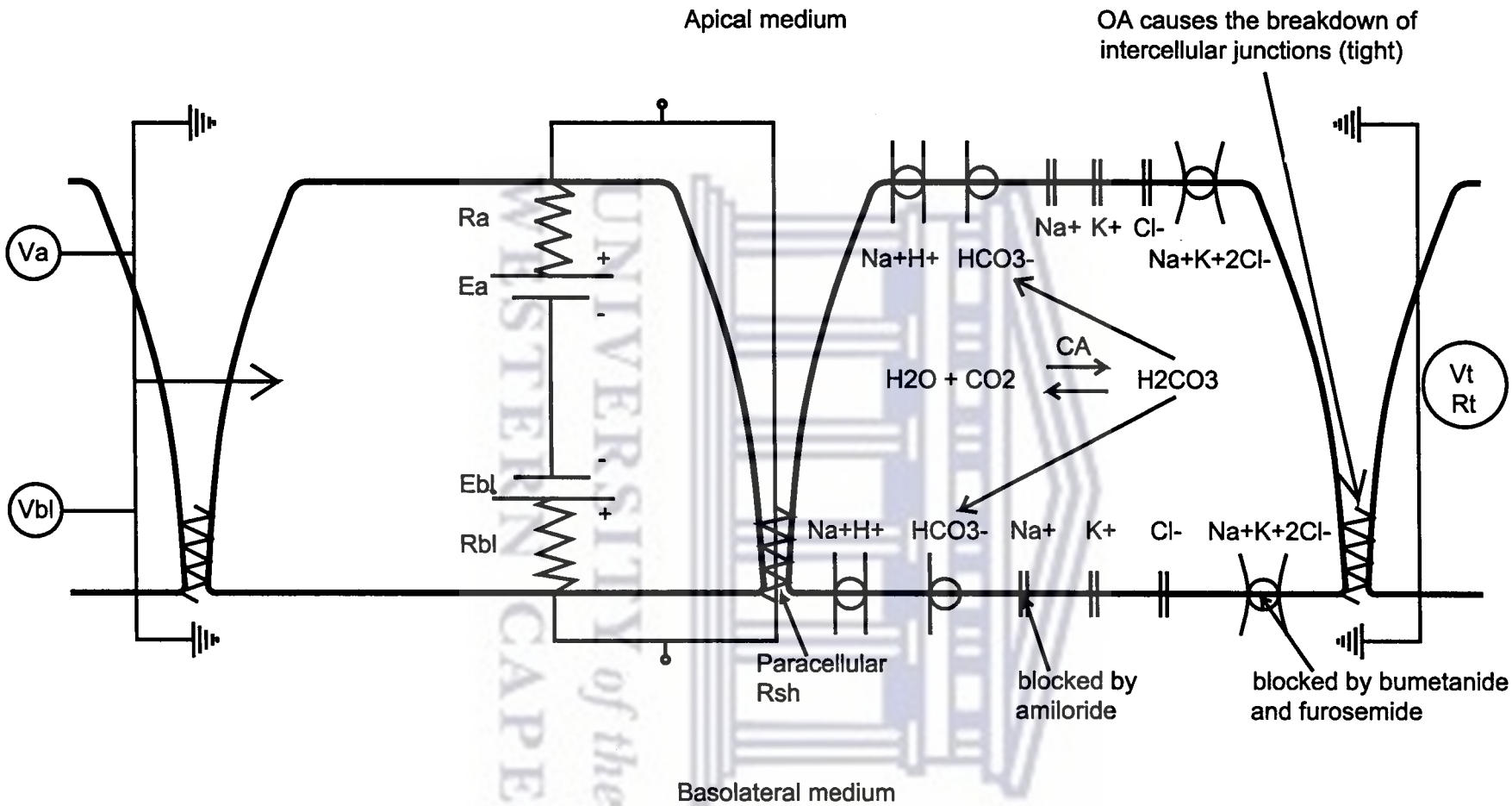


Fig. 5.1 The Electrical equivalent circuit and graphical representation of Ionic transport mechanisms across TM4 confluent monolayers as found in this study

REFERENCES

1) **Baldrick, P., Hyde, D. and Anstee, H.** (1988). Microelectrode studies on Malpighian tubule cells of *Locusta migratoria*: effects of external ions and inhibitors. *Journal of Insect Physiology*. 34(10): 963-975.

2) **Bardin, C.W., Cheng, C.Y., Mustow, N.A. and Gunsalus, G.L.** (1994). The Sertoli cell. The *Physiology of Reproduction. Second Edition. Edited by E. Knobil and J.D. Neil.* Raven Press, NY.1291-1333.

3) **Bennet, M.**, De Kock, M. and van Der Horst, G. (2000). Oleanolic acid induces apoptosis in TM4 Sertoli cells. *Poster presentation, 8th International Symposium of Spermatology.*

4) **Berne, R.M. and Levy, M.N.** (1993). *Principles of Physiology*. Mosby Inc. 520-600.

5) **Berne, R.M. and Levy, M.N.** (2000). *Principles of Physiology*.3rd edition. Mosby Inc. 590-595.

6) **Brinster, R.L. and Zimmermann, J.W.** (1994). Spermatogenesis following male germ-cell transportation. *Proc. Natl. Acad. Sci. USA*. 91: 11298-11302.

7) **Cheng, C.Y., Grima, J., Stahler, M.S., Guglielmotti, A., Silvestrini, B. and Bardin, W.** (1990). Sertoli Cell Synthesizes and Secretes a Protease Inhibitor, 2-Macroglobulin. *Biochemistry*. 29:1063-1068.

8) **Clauss, W., Hoffmann, B., Krattenmacher, R. and Van Driessche, W.** (1993). Current noise analysis of Na absorption in the embryonic coprodeum: stimulation by aldosterone and thyroxine. *American Physiological Society*. Available:
www.tzm.unigiessen.de/unigi/layout/englisch

9) **Cuthbert, A.W. and Wong, P.Y.D.** (1975). Intracellular potentials in cells of the seminiferous tubules of rats. *Journal of physiology*. 248: 173-191.

10) **Davis, J.C. and Bordy, M.** (1980). FSH stimulation of active, cell-specific aggregation by immature Sertoli cells in primary culture. *Testicular Development, Structure, and Function,* edited by A. Steinberger and E. Steinberger, Raven Press, New York. 79-87.

11) **D'agostino, A., Mene, P. and Stefanni, M.** (1992). Voltage-gated calcium channels in rat Sertoli cells. *Biology of Reproduction*. 46: 414-418.

12) **De Kock, M.** (1989). Some effects of gammalinolenic acid on the mitotic indices and ultrastructure of malignant MG63 osteogenic sarcoma cells in culture. *M.Sc Thesis*. MEDUNSA. Pretoria. South Africa.

13) **Dorrington, J.H. and Fritz, I.B.** (1974). Effects of gonadotropins on cyclic AMP production by isolated seminiferous tubule and interstitial cell preparations. *Endocrinology*. 94:395-403.

14) **Eusebi, F., Ziparo, E., Fratamico, G., Russo, M.A.** (1983). Intercellular Communication in Rat Seminiferous Tubules. *Developmental Biology*. 100: 249-255.

15) **Foresta, C., Rossato, M., Bordon, P. and Di Virgilio, F.** (1995). Extracellular ATP activates different signalling pathways in rat Sertoli cells. *Biochem. J.* 311: 269-274.

16) **Fisher, D.** (1997). An exploration of the electrophysiological property and ion transport across the isolated seminiferous tubule of the rat. *Thesis*. University of the Western Cape. Bellville. South Africa. 7535.

17) **Fisher, D.** (2002). New light shed on the seminiferous tubule. *Journal of Physiology*. 542.2: 445-452.

18) **Fox, S.I.** (1996). *Human Physiology*. 5th edition. WCB Publishers. 122-140 and 608-628.

19) **Ganong, W.F.** (1983) *Review of Medical Physiology.* 11th edition. Lange Medical Publications. 1-29.

20) **Garcia-Granados, A., Martinez, A., Moliz, J.N., Parra, A. and Rivas, F.** (1998). Molecules. [Online]. Available http://www.mdpi.org/molbank/m0087.htm

21) **Granger, S and Scott, I.** (1998). Skin care compositions containing a polycyclic triterpene carboxylic acid and a retenoid. United States patent no. 5,723,139. Available: www.amsar.com/oleanolic.htm

22) **Gerard, H., Gerard, A., Nya, A.E., Felden, F. and Gueant, J.L.** (1994). Spermatogenic Cells Do Internalize Sertoli Cell Androgen-Binding Protein: A Transmission Electron Microscopy Autoradiographic Study in the Rat. *Endocrinology.* 134(3): 1515-1527.

23) **Gladwell, R.T.** (1977). The effect of temperature on the potential difference and input resistance of rat seminiferous tubules. *J. Physiol.* 268: 111-121.

24) **Gow, A., Southwood, C.M., Li, J.S., Pariali, M., Riordin, G.J., Brodie, S.E., Danias, J., Bronstein, J.M., Kachar, B. and Lazzarini, R.A.** (1999). CNS Myelin and Sertoli Cell Tight Junction Strands Are Absent in *Osp/Claudin-11* Null Mice. *Cell.* 99: 649-659.

25) **Hadley, M.A., Byers, S., Suarez-Quian, C.A., Kleinman, H.K. and Dym, M.** (1985). Extacellular matrix regulates Sertoli cells differentiation, testicular cord formation and germ cell development *in vitro. Journal of cell Biology.* 101: 1511-1522

26) **Hamill, O.P., Marty, A., Neher, E., Sakmann, B. and Sigworth, F.J.** (1981). Improved Patch-Clamp Techniques for High-Resolution Current Recording from Cells and Cell-Free Membrane Patches. *Pflugers* Arch. 391: 85-100.

27) **Hanrahan, J.W., Wills, N.K., Phillips, J.E. and Lewis, S.A.** (1986). Channel Density, Conductance, and Block by Barium. *J.Gen.Physiol.* 87:443-466.

28) **Harborne, J.H.** (1983). Phytochemical methods. WCB Publishers.

29) **Harkness J.E. and Wagner. J.** (1996) The biology and medicine of rabbits and rodents 3rd Ed.

30) **Hoffmann D.L.** (2002). Herbs, Fertility and Contraception. [Online]. Available http://www.stopgettingsick.com/Conditions/condition_template.cfm/1382/69/16

31) **Janecki, A., Jakubowiak, A. and Steinberger, A.** (1990). Vectorial Secretion of Inhibin by Immature Rat Sertoli Cells in vitro: Re-examination of Previous Results. *Endocrinology.* 127: 1896-1904.

32) **Janecki, A., Jakubowiak, A. and Steinberger, A.** (1991). Regulation of transepithelial resistance in two-component Sertoli cell cultures: In vitro model of the Blood-Testis Barrier. *Endocrinol.* 129: 1489-1496.

33) **Janecki, A., Jakubowiak, A. and Steinberger, A.** (1992). Effect of Cadmium chloride on transepithelial electrical resistance of Sertoli cell monolayers in two-compartment cultures-A new model for toxicologicalinvestigationsof the "blood-testis" barrier *in vitro. Toxicology and Applied Pharmacology.* 112: 51-57.

34) **Jegou, B.** (1992). The Sertoli cell. *Bailliere's Clinical Endocrinology and Metabolism.* 6.2: 273-300.

35) **Joffre, M., Roche, A. and Duchatelle, P.** (1988). Electrophysiological studies of the action of gonadotropins on Leydig and Sertoli cells from rat testis. *Reprod.Nutr.Develop.* 28 (4b): 1019-1029.

36) **Jungwirth, A., Weiger, T., Singh, S.K., Paulmichl, M. and Frick, J.** (1997). Follicle-Stimulating Hormone activates a cAMP-dependant chloride conductance in TM4 Sertoli cells. *Biochemical and Biophysical Research Communications*. 233: 203-206.

37) **Kapil, A. and Sharma, S.** (1995). Effect of Oleanolic Acid on Complement in Adjuvant- and Carrageenan-induced Inflammation in Rats. *J. Pharm. Pharmacol.* 47: 585-587.

38) **Ko, W.H., Chan, H.C., Chew, S.B. and Wong, P.Y.D.** (1998). Regulated anion secretion in cultured epithelia from Sertoli cells of immature rats. *Journal of Physiology*. 512.2: 471-480.

39) **Kong, Y.C., Xie, J. and But, P.P.** (1986). Fertility Regulating Agents from Traditional Chinese Medicines. *Journal of Ethnopharmacology*. 15: 1-44.

40) **Lalevee, N. and Joffre, M.** (1999). Inhibition by cAMP of Calcium-Activated Chloride Currents in Cultured Sertoli Cells from Immature Testes. *J. Membrane Biol.* 169. 167-174.

41) **Levine, N. and Marsh, D.J.** (1971). Micropuncture studies of the electrochemical aspects of fluid and electrolyte transport in individual seminiferous tubules, the epididymis and the vas deferens in rats. *Journal of Physiology*. 213: 557-570.

42) **Levine, N. and Marsh, D.J.** (1975). Micropuncture study of the fluid composition of 'Sertoli cell-only' seminiferous tubules in rats. *J. Reprod. Fertil.* 43(3): 547-549.

43) **Liu, J.** (1995). Pharmacology of oleanolic acid and ursolic acid. *Journal of Ethnopharmacology*. 49: 57-68.

44) **Lue, Y., Sinha Hikim, A.P., Wang, C., Im, M., Leung, A. and Swerdloff, R.S.** (2000). Testicular heat exposure enhances the suppression of spermatogenesis by testosterone in rats: the "two-hit" approach to male contraceptive development. *Endocrinology*. 141.4: 1414-1424.

45) **Maren, P.** (1967) Intra-cellular buffering of hydroxyl ions produced during hydrogen ion secretion by the parietal cells of the gastric mucosa [ONLINE] visit: www.ijp-online.com/archives/1981/ 013/04/r0357-0361ra.pdf

46) **Mather, J.P.** (1980). Establishment and characterisation of two distinct mouse testicular epithelial cells lines. *Biol. Reprod.* 23: 245-252.

47) **Matthews, C and Van Holde, K** (1992) *Biochemistry. 3rd Edition*. Mosby Inc. 680-726.

48) **Mdhluli, M.C. and van der Horst, G. (2002)** The effect of oleanolic acid on sperm motion characteristics and fertility of male Wistar rats. *Laboratory animals*. 36 (4) : 438-444.

49) **Meehan, T., Schlatt, S., O'Bryan, M.K., de Kretser, D.M. and Loveland, K.L.** (2000). Regulation of Germ Cell and Sertoli Cell Development by Activin, Follistatin, and FSH. *Developmental Biology*. 220: 225-237.

50) **Mills, N.C.** (1990). Androgen effects on Sertoli cells. *International Journal of Andrology*. 13:123-134.

51) **Muto, Y., Ninomiya, M. and Fujiki, H.**(1990). Present status research in cancer chemoprevention in Japan. *Japanese Journal of Clinical Oncology*. 20: 219-224.

52) **Onoda, M., Suarez-quian, C.A., Djakiew, D. and Dym, M.** (1990). Characterization of Sertoli cells cultured in the bicameral chamber system: Relationship between formation of permeability barriers and polarized secretion of transferrin. *Biology of Reproduction*. 43: 672-683.

53) **Palfrey, H.C. and Rao, M.C.** (1983). Na/K/Cl co-transport and if regulation. *J. Exp. Biol.* 106: 43-54.

54) **Parvinen, M., Marana, R., Robertson, D.M., Hansson, V. and Ritzen, E.M.** (1980). Functional cycle of rat Sertoli cells: differential binding and action of Follicle Stimulating Hormone at varios stages of the spermatogenic cycle. *Testicular Development, Structure, and Function,* edited by A. Steinberger and E. Steinberger, Raven Press, New York. 425-432.

55) **Parvinen, M. and Ruokonen, A.** (1982). Endogenous steroids in the rat seminiferous tubules. Comparison of the epithelial cycle isolated by Transillumination- assisted microdissection. *Journal of Andrology*. 3: 211-220.

56) **Phillipson., J D**. (2001). Phytochemistry and medicinal plants. *Phytochemistry*. 56: 237-243.

57) **Raivio, T., Saukkonen, S., Jaaskelainen, J., Kormulainen, J. and Dunkel, L.** (2000). Signalling between the pituitary gland and the testes: inverse relationship between serum FSH and inhibin B concentrations in boys in early puberty. *Journal of Endocrinology*. 142: 150-156.

58) **Rajasekaran, M., Bapna, J.S., Lakshmanan, S., Ramachandran Nair, A.G., Veliath, A.J. and Panchanadam, M.** (1988). Antifertility effect in male rats of oleanolic acid, a triterpene from Eugenia jambolana flowers. *Journal of Ethnopharmacology*. 24: 115-121.

59) **Rhoades, R and Pflanzer, R.** (1996). *Human Physiology*. 3rd edition. Saunders College Publishing. 712-720.

60) **Roche, A. and Joffre, M.** (1989). Effect of uncoupling treatments on FSH-induced hyperpolarization of immature rat Sertoli cells from Sertoli cell enriched cultures. *J. Reprod.* 85: 343-354.

61) **Russell, L., Myers, P., Ostenburg, J. and Malone, J**. (1980). Sertoli Ectoplasmic Specializations During Spermatogenesis. *Testicular Development, Structure and Function*. Raven Press, N.Y. 55-63.

62) **Setchell, B.P.** (1979). The functional significance of the blood-testis barrier. *Journal of Andrology*. 1: 3-10.

63) **Sherwood, L.** (2001). Human Physiology: From cells to systems. 4th edition. WCB. 368-386.

64) **Singh, S.K., Binder, H.J., Geibel, J.P. and Boron, W.F.** (1995). An apical permeability barrier to NH3/NH4+ in isolated, perfused colonic crypts. *Physiology*. 92: 11573-11577.

65) **Singh, G.B., Singh, S., Bani, S., Gupta, B.D. and Banerjee, S.K.** (1992). Anti-inflammatory activity of oleanolic acid in rats and mice. *Journal of Pharmacy and Pharmacology*. 44: 456-458

66) **Tcholakian, R.K. and Steinberger, A.** (1980). In Vitro Metabolism of Testosterone by Sertoli Cells and Interstitial Cells and the Effect of FSH. *Testicular Development, Structure and Function*. Raven Press, N.Y. 177-183.

67) **Thibodeau, G.A. and Patton, K.T.** (1996). The male reproductive system. *Amatomy and Physiology*. 3rd edition: 996-1003.

68) **Tortora, G.J. and Anagnostakos, N.P.** (1987). The reproductive systems. *Principles of Anatomy and Phyisiology*. 5th edition: 705-713.

69) **Toppari, J., Vihko, K.K., Rasanen, K.G.E., Eerola, E. and Parvinen, P.** (1986). Regulation of stages VI and VIII of the rat seminiferous epithelial cycle. *Journal of Endocrinology*. 108: 417-422.

70) **Tindall, D.J., Vitale, R. and Means, A.R.** (1975). Androgen binding protein as a biochemical marker of formation of the blood testis barrier. *Endocrinology*. 97: 636-648.

71) **Tokuda, H., Ohigashi, H., Koshimizu, K. and Ito, Y.** (1986). Inhibitory effects of ursolic acid and oleanolic acid on skin tumor promotion by 12-O-tetradecanoylphorbol-13 –acetate. *Cancer letters*.33: 279-285

72) **Tuck, R.R., Setchell, B.P., Waites, G.M.H. and Young, J.A.** (1970). The composition of fluid collected by micropuncture and catheterization from the seminiferous tubules and rete testis of rats. *Pflugers Arch*. 318: 225-243.

73) **Van Damme, M.P., Robertson, D.M., Marana, R., Ritzen, E.M. and Diczfalusy, E.** (1980). Aromatization of Androgens by Sertoli Cells: A Sensitive and Specific Bioassay for FSH Activity. *Testicular Development, Structure and Function*. Raven Press, N.Y. 169-175.

74) **Verhoeven, G.** (1999). Spermatogenesis and spermatogenic control: a state of the art. *K Acad Geneeskd Belg*. 61(3): 417-432

75) **Waites, G.M.H. and Gladwell, R.T.** (1982). Physiological significance of fluid secretion in the testis and blood-testis barrier. *Physiological Reviews*. **62**: 624-671.

76) **Weltens, R., Leyssens, A., Zhang, S.L., Lohrmann, E., Steels, P. and van Kerkhove, E.** (1992). Unmasking of the Apical Electrogenic H Pump in Isolated Malpighian Tubules (Formica polyctena) by the use of Barium. *Cell Physiol Biochem*. 2:101-116.

77) **Xu, S.L.** (1985). Effects of oleanolic acid on chronic hepatitis: 188 case reports. *Symposium on oleanolic acid*. 23-25.

78) **Van Wyk, B.E., van Oudtshoorn, B. and Gericke, N.** (1997) *Medicinal plants of South Africa* .Briza Publications.

79) **Wasserman, G.F., Monti Bloch, L., Grillo, M.L., Silva, F.R.M.B., Loss, E.S. and McConnell, L.L.** (1992). Electrophysiological changes of Sertoli cells produced by the acute administration of amino acid and FSH. *Horm. Metab. Res*. 24:326-328.

APPENDICES

Appendix A

Dulbecco's Modified Eagle Medium (DMEM) Liquid 12-604

Component	*MG/L Inorganic Salts*
$CaCl_2$ (anhydrous)	200.00
$Fe(NO_3)_3.9H2O$	0.10
KCl	400.00
$MgSO_4$.$7H_2O$	200.00
NaCl	6,400.00
$NaHCO_3$	3,700.00
NaH_2PO_4 .H_2O	125.00
Other Components	
Glucose	4,500.00
Phenol Red.Na	15.93
Sodium Pyruvate	110.00
Amino Acids	
L-Arginine.HCl	84.00
L-Cystine	48.00
L-Glutamine	584.00
Glycine	30.00
L-Histidine.HCl	42.00
L-lsoleucine	104.80
L-Leucine.HCI	104.80
L-Methionine	146.20
L-Phenylalanine	30.00

L-Serine	66.00
L-Threonine	95.20
L-Tryptophan	16.00
L-Tyrosine	72.00
L-Valine	93.60
Vitamins D(+)-Ca Pantothenate	4.00
Choline Chloride	4.00
Folic Acid	4.00
i-Inositol	7.00
Nicotinamide	4.00
Pyridoxine.HCl	4.00
Riboflavin	0.40
Thiamine.HCl	4.00
Storage Temperature	2-8°C

Appendix B

Determining the concentration of oleanolic acid in crude extracts

Once the crude extracts had been immersed in ethanol (see chapter 2), High Pressure Liquid Chromatography (HPLC) was performed to determine the concentration of OA in the extracts.

The chromatographic system used was a Beckman HPLC system consisting of a double pump Programmable Solvent Module 126, Diode Array Detector module 168, with 32 Karat Gold software supplied by Beckman; Column C18 Bondapak 5 μm dimensions (250 x 4.6mm). The chromatographic conditions were as follows: Mobile phase, solvent A: Ethanol (EtOH); solvent B: 5% acetic acid (CH_3COOH); Mode: gradient, increasing the organic phase (EtOH) from 20% to 90% over 18 minutes; flow rate: 1ml/min.; reference standard: OA (1g dissolved in 100ml EtOH); OU and OX extracts used in the direct bioassay. Injected volume5μl. The run time was 25 min.

From this procedure, the concentration of oleanolic acid was determined to be 10mg/ml in OU and 7mg/ml in OX.